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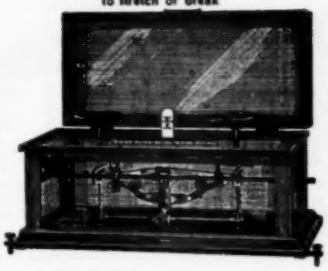

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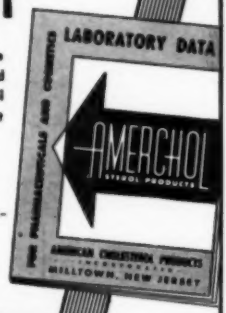

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Observations on the Solubility of Certain Sulfonamides*,†

ALFRED R. BIAMONTE‡ and GEORGE H. SCHNELLER§

The solubility of a triple sulfonamide mixture consisting of equal parts of sulfadiazine, sulfamerazine, and sulfamethazine, and of a mixture of equal parts of their N^4-acetyl derivatives was determined in the range of pH 4.5 to 7.5 at 37°, utilizing sodium phosphate-citric acid buffer solutions as solvent. The solubility of N^1-(3,4-dimethyl-5-isoxazolyl) sulfanilamide and its N^4-acetyl derivative was also determined. A comparison of the solubility data is made and is discussed briefly. Details of the experimental and analytical procedures are described.

MIXED sulfonamide therapy has been accepted by medical authorities for several years and very large quantities of sulfonamides are now used in the form of mixtures of two or more—most often, three—individual components.

Sulfadiazine and sulfamerazine are invariably present in modern sulfonamide combinations. Sulfathiazole was formerly widely used as the third component, but more recently sulfamethazine (the dimethyl derivative of sulfadiazine) has achieved a dominant position in this field. All of the triple sulfonamide combinations recognized in New and Nonofficial Remedies (1), published by the Council on Pharmacy and Chemistry of the American Medical Association, contain sulfamethazine as the third component.

In the clinical use of sulfonamide mixtures instead of a single compound, the principal object is to minimize, if not eliminate, the risk of crystals of the sulfonamide or its N^4-acetyl derivative being deposited in the kidney with resulting damage or actual block. This effect is due to the simple chemical fact of additive solubilities. A number of factors (some of them imponderable) are involved in the question whether a given sulfonamide or combination of sulfonamides is likely to crystallize in the kidneys of a particular human patient. But the solubility of the compound or of the combination is one of the most important factors and this is one that is capable of precise determination. Therefore, it has been the custom to assign considerable importance in the evaluation of a sulfonamide or combination of sulfonamides, to the solubility as determined *in vitro*.

Lehr (2), in the course of his early work in this field, determined the solubilities of a considerable number of combinations of two, three, and even four sulfonamides, but his series did not include a combination of sulfadiazine, sulfamerazine, and sulfamethazine. Frisk (3) and his coworkers reported the solubility of a combination of sulfadiazine, sulfamerazine, and sulfathiazole

* Received January 5, 1952, from the American Cyanamid Co., Calco Chemical Division, Bound Brook, N. J.
† Presented at the Joint Session of the Subsection on Pharmacy, American Association for the Advancement of Science and the AMERICAN PHARMACEUTICAL ASSOCIATION, Philadelphia, Pa., December 27, 1951.
‡ Pharmaceutical Application Laboratory, American Cyanamid Co., Calco Chemical Division.
§ Director, Pharmaceutical Application, American Cyanamid Co., Calco Chemical Division.

at a series of *p*H levels within the range 5.9 to 7.9 but, again, did not work with a combination of the three sulfapyrimidines.

Since the sulfonamide combination most frequently prescribed currently consists of sulfadiazine, sulfamerazine, and sulfamethazine, it was considered desirable to determine the solubility of this combination at various *p*H levels within the physiologic range encountered in the urine. It was also considered interesting to compare these solubility data with data obtained under the identical experimental conditions with a sulfonamide of the "soluble" type. Aqueous buffers were used as the solvent rather than urine, for the sake of convenience and because it has been shown (12) that the solubility of these sulfonamides in phosphate buffer closely approximates that in urine.

It was, therefore, the purpose of this investigation to determine the solubility of a combination of sulfadiazine, sulfamerazine, and sulfamethazine and also of their N^4-acetyl derivatives in aqueous buffer solutions at 37° over the physiologically significant *p*H range. A further purpose of this investigation was to determine the solubility of N^1-(3,4-dimethyl-5-isoxazolyl) sulfanilamide and of its N^4-acetyl derivative under the same experimental conditions. The latter sulfonamide is considered a "soluble" compound (4, 5), but no data have appeared in the literature comparing its solubility with that of a widely prescribed combination of sulfonamides determined under similar conditions.

EXPERIMENTAL

A triple sulfonamide mixture containing equal portions of sulfadiazine, sulfamerzine, and sulfamethazine was prepared.[1] A mixture, containing equal parts of acetylsulfadiazine, acetylsulfamerazine, and acetylsulfamethazine was also prepared.[2] The solubility determinations for both triple sulfonamide mixtures were carried out on these preparations. A sample large enough to supply an excess of each constituent was used to insure saturation of each sulfonamide in solution.

The solubility determinations were performed as follows: 500 mg. of the sulfonamide compound or mixture were placed in a 125-ml. ground joint Erlenmeyer flask containing several glass beads. Fifty milliliters of sodium phosphate-citric acid buffer [McIlvaine's (6)] of the desired *p*H were added. For the solubility determinations at *p*H 7 and higher, 1.5 Gm. of sulfonamide per 50 ml. of buffer solvent were used. Using a glass tube having a ground joint on one end with a rubber stopper on the other end, the flask was sealed and attached to a stirrer so arranged that the flask both rotated and agitated the solution while submerged in water maintained at 37°. The flasks were agitated for eighteen hours. At the end of this period the suspensions were immediately filtered by gravity through Whatman No. 1 paper (9 cm.). Filtration time was approximately two minutes. The *p*H of the sulfonamide suspension and the filtrate were carried out at 37°. The filtrate was collected in a 25-ml. volumetric flask. Further dilution was necessary before the analysis for sulfonamide content was performed. The solubility determinations were carried out in duplicate and each test for solubility was analyzed in duplicate.

ANALYTICAL PROCEDURE

The procedure employed for the determination of sulfonamides was based upon the method developed by Bratton and Marshall (7).

Reagents

(*a*) Sodium nitrite, 100 mg. per 100 ml. distilled water.

(*b*) Ammonium sulfamate, 0.5 Gm. per 100 ml. distilled water.

(*c*) N-(1-naphthyl)-ethylenediamine dihydrochloride, 100 mg. per 100 ml. distilled water.

(*d*) 1 *N* HCl.

(*e*) Stock solutions of sulfonamides, 200 mg. (total sulfonamide) per liter.

Dilute standard solutions were prepared using 2, 5, 10, 15, 20, 25, and 30-ml. portions of the stock solution. Each of these aliquots was diluted to 100 ml. An aliquot of 5 ml. from each standard solution was transferred to separate 100-ml. volumetric flasks and 25 ml. of water, 10 ml. of 1 *N* HCl, and 10 ml. of each reagent (*a*, *b*, and *c*) were added. After the addition of sodium nitrite, the flasks were permitted to stand for three minutes with intermittent shaking. The solutions were diluted to 100 ml., mixed, allowed to stand for ten minutes, and the color produced was read in a Beckman DU spectrophotometer at wave length 545. A blank prepared with the reagents was employed. The standard curve was plotted from these readings. Standard curves were prepared for the triple sulfonamide mixture and for N^1-(3,4-dimethyl-5-isoxazolyl)sulfanilamide. These curves were also used for the triple acetylsulfonamide mixture and for acetyl-N^1-(3,4-dimethyl-5-isoxazolyl)sulfanilamide, respectively, with appropriate conversion factors based upon the molecular weights of these compounds. The solubility values so obtained for the triple sulfonamide mixture and the triple acetyl sulfonamide mixture deviate slightly from the actual values, since the standard curve was prepared from a mixture of equal parts of three components, whereas different ratios prevailed in the solutions under measurement because of the differences in the individual solubilities. These deviations are negligible.

The method just described was applied directly to solutions containing triple sulfonamide mixtures and to those containing N^1-(3,4-dimethyl-5-isoxazolyl) sulfanilamide. However, in order to determine the acetylated compounds, it was necessary

[1] Melting Points: sulfadiazine, 253.5–254.5; sulfamerazine, 235.5–236.5; sulfamethazine, 197.7–198.6; N^4-acetylsulfadiazine, 261.2–262.4; N^4-acetylsulfamerazine, 248.2–249.4; N^4-acetylsulfamethazine, 249.5–250.6; N^1-(3,4-dimethyl-5-isoxazolyl) sulfanilamide, 193.4–193.9; N^1-(3,4-dimethyl-5-isoxazolyl) N^4-acetylsulfanilamide, 214.8–215.9.

[2] The acetyl derivatives were supplied by Dr. J. M. Smith, Jr., American Cyanamid Co., Calco Chemical Division, Bound Brook, N. J.

to hydrolyze these compounds before proceeding with the analysis. Acid hydrolysis of triple acetyl sulfonamide mixtures gave satisfactory results but acid hydrolysis of acetyl-N¹-(3,4-dimethyl-5-isoxazolyl) sulfanilamide produced side reactions indicated by color interference formed upon subsequent diazotization and coupling. Satisfactory results for the determination of acetylated N¹-(3,4-dimethyl-5-isoxazolyl) sulfanilamide were obtained after subjecting the compound to alkaline hydrolysis.

During the course of this work, it was believed that possibly hydrolysis of the acetyl sulfonamide compounds may occur during the eighteen-hour saturation period, particularly at the different *p*H levels. Therefore, for the determination of the concentration of the acetylated compounds in solution, both the total and free sulfonamides were determined. The difference between the total and free sulfonamide represents the acetylated sulfonamide. The degree of hydrolysis occurring during the solubility determination was negligible.

Procedure for Triple Sulfonamide Mixture and N¹ - (3,4 - Dimethyl - 5 - isoxazolyl) Sulfanilamide.—Transfer an aliquot representing approximately 0.10 to 0.25 mg. of the triple sulfonamide mixture to a 100-ml. volumetric flask. Add 10 ml. of 1 *N* HCl and 30 ml. of water. Add 10 ml. of the sodium nitrite and mix for three minutes. Add 10 ml. of ammonium sulfamate solution and mix for two minutes. Add 10 ml. of the N-(1-naphthyl)-ethylenediamine dihydrochloride solution and dilute with water to volume. Mix and let stand for ten minutes before reading the color. For the blank, use the reagents and diluent used for the test, omitting the sulfonamide solution.

Procedure for Triple Acetyl Sulfonamide Mixture.—Determine the total sulfonamides as follows: treat an aliquot portion of a solution representing 2–5 mg. of the triple acetyl sulfonamide mixture with 5 ml. of concentrated HCl, dilute with water to 40 ml. total volume, and boil for fifteen to twenty minutes. Cool and transfer to a 100-ml. volumetric flask and dilute to volume. Transfer a 5-ml. aliquot to a 100-ml. volumetric flask and continue as in the above procedure for triple sulfonamide mixture and N¹-(3,4-dimethyl-5-isoxazolyl) sulfanilamide, starting with: add 10 ml. of 1 *N* HCl, etc.

Determine free sulfonamide exactly as in the procedure for triple sulfonamide and N¹-(3,4-dimethyl-5-isoxazolyl) sulfarilamide.

Subtract free sulfonamide from total sulfonamide. This difference, when multiplied by the appropriate conversion factor, represents the amount of acetyl sulfonamide in the diluted solution. Applying the dilution factor gives the concentration of acetyl sulfonamide in the original solution.

Procedure for Acetylated N¹(3,4-Dimethyl-5-isoxazolyl) Sulfanilamide.—Determine total sulfonamides as follows: treat an aliquot representing 2-6 mg. of acetyl-N¹-(3,4-dimethyl-5-isoxazolyl) sulfanilamide with 5 ml. of sodium hydroxide 30% solution, dilute with water to 40 ml. and boil for fifteen to twenty minutes. Cool and dilute to 100 ml. in a volumetric flask. Transfer a 5-ml. aliquot to a 100-ml. volumetric flask, add 15 ml. 1 *N* HCl and 25 ml. of water, and continue as in procedure for triple sulfonamide mixture and N¹-(3,4-dimethyl-5-isoxazolyl) sulfanilamide starting with: add 10 ml. sodium nitrite solution, etc.

Determine free sulfonamide exactly as in the procedure for triple sulfonamide mixture and N¹-(3,4-dimethyl-5-isoxazolyl) sulfanilamide.

The difference between the results obtained for total and free sulfonamides represents the concentration of acetylated N¹-(3,4-dimethyl-5-isoxazolyl) sulfanilamide in solution after applying the appropriate conversion and dilution factors.

Accuracy of Method.—The accuracy of the method was established as follows: aliquots of 10, 15, 20, and 25 ml. of sulfonamide stock solution were taken and analyzed for their sulfonamide content. The results obtained for triple sulfonamide mixture and N¹-(3,4-dimethyl-5-isoxazolyl) sulfanilamide are given in Table I.

TABLE I.—ACCURACY OF ANALYTICAL METHOD

Triple Sulfonamide Mixture Mg. per 100 Ml.		N¹-(3,4-Dimethyl-5-isoxazolyl) Sulfanilamide Mg. per 100 Ml.	
Expected	Found	Expected	Found
2.000	2.02	2.003	2.08
3.000	3.04	3.004	3.06
4.000	4.06	4.006	4.12
5.000	5.04	5.007	5.10

A similar degree of precision was obtained in the analysis of the triple acetyl sulfonamide mixture and of acetyl N¹-(3,4-dimethyl-5-isoxazolyl) sulfanilamide.

As a further measure of control, a sample representing a known quantity of the specific sulfonamide compound or mixture was analyzed with each analysis performed on the solubility test solutions.

RESULTS

The solubilities of the sulfadiazine-sulfamerazine-sulfamethazine mixture, of N¹-(3,4-dimethyl-5-isoxazolyl) sulfanilamide, and of their N⁴-acetyl derivatives are given in Table II and graphically in Fig. 1. Frisk has shown that the principle of additive solubilities applies to mixtures of sulfonamides with their N⁴-acetyl derivatives. Therefore the values for the total sulfonamide concentration representing the calculated sum of the solubilities of both free and acetylated sulfonamides are also given.

The solubility of the triple sulfonamide mixture is greater than that of N¹-(3,4-dimethyl-5-isoxazolyl) sulfanilamide at *p*H levels below 5.5. The solubility of the triple sulfonamide mixture slowly increases from 95.9 mg./100 ml. at *p*H 4.5 to 108.9 mg./100 ml. at *p*H 5.8, whereas the solubility of the isoxazole compound increases rapidly from 32.3 mg./100 ml. at *p*H 4.5 to 262.0 mg./100 ml. at *p*H 5.9. At about *p*H 6.0 the solubility of N¹-(3,4-dimethyl-5-isoxazolyl) sulfanilamide is more than twice that of the triple sulfonamide mixture and at approximately *p*H 6.8 the solubility is more than tenfold. On the other hand, at *p*H 4.5, the solubility of the isoxazole derivative is one-third that of the triple sulfonamide combination. The acetyl derivative of N¹-(3,4-dimethyl-5-isoxazolyl) sulfanilamide is less soluble than the parent compound, whereas the acetylated triple sulfonamide mixture is more soluble than the parent mixture. For example, the solubility of the acetylsulfonamide mixture is 119.5 and 152.7 mg./100 ml. at *p*H 4.5 and 6.0, respectively, whereas for

FIG. 1

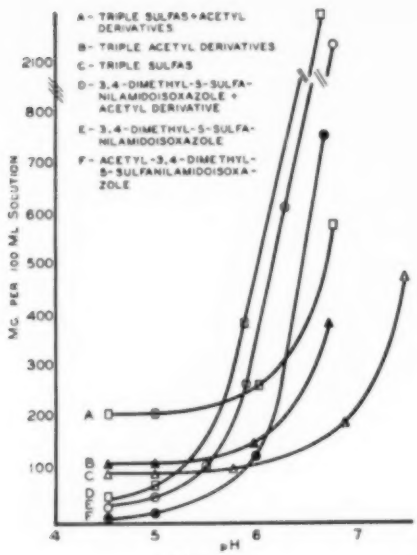

Figure 1.

Solubility of triple sulfonamide mixture, of N^1-(3,4-Dimethyl-5-isoxazolyl) sulfanilamide, and their N^4-acetyl derivatives at 37° in sodium phosphate-citric acid buffer solutions.

Equilibrium time: 18 hr.

the acetylated isoxazole sulfonamide, the solubility is 6.0 and 126.1 mg /100 ml. at the corresponding *p*H levels.

The solubility of the acetyl-isoxazole compound approaches that of the acetylated triple sulfonamide mixture at approximately *p*H 6.1. At *p*H levels below 6.1, the solubility of the triple acetyl-sulfonamide mixture is greater than that of the acetyl-isoxazole sulfonamide, whereas at *p*H above 6.1 the solubilities are reversed.

The total sulfonamide solubility, as shown in Table II and Fig. 1, represents the calculated sum of the acetylated sulfonamides. Below approximately *p*H 5.7 the total sulfonamides (free and combined) in solution (represented by the calculated sum of the observed solubilities of the triple sulfonamide mixture and of the acetylated mixture) are greater than the sum of the observed solubilities of N^1-(3,4-dimethyl-5-isoxazolyl) sulfanilamide and its acetylated form, whereas at *p*H levels greater than 5.7 the reverse is true.

Figure 2 shows the individual solubilities of the sulfapyrimidines as reported in the literature (11). The totals of these are in satisfactory agreement with the total solubilities determined in this study.

DISCUSSION

While a full discussion of the clinical significance of these data is beyond the scope of this report, one important implication appears to be worthy of mention. This is, that the combination of sulfadiazine, sulfamerazine, and sulfamethazine deserves to be considered a "soluble" sulfonamide combination since, in the most critical portion of the physiological *p*H range, its solubility exceeds that of a sulfonamide which is generally termed "soluble."

This comparison affords an interesting example of the applicability of chemical data to the solution of a clinical problem. While the urine of normal humans is usually considered to have a mean *p*H value of 6, it ranges (8) as low as *p*H 4.8, and in

TABLE II.—SOLUBILITY OF N^1-(3,4-DIMETHYL-5-ISOXAZOLYL) SULFANILAMIDE AND ITS ACETYL DERIVATIVE COMPARED WITH THAT OF A MIXTURE OF EQUAL PARTS OF SULFADIAZINE, SULFAMERAZINE, AND SULFAMETHAZINE AND THEIR ACETYL DERIVATIVES

	Solubility in Sodium Phosphate-Citric Acid Buffers at 37° (Mg. per 100 Ml. of Solution)[a]				
Initial *p*H of Buffer	N^1-(3,4-Dimethyl)-5-isoxazolyl) Sulfanilamide A	Final *p*H at 37°	N^1-(3,4-Dimethyl-5-isoxazolyl) N^4-Acetyl Sulfanilamide B	Final *p*H at 37°	Total Sulfonamides Columns A + B
4.5	32.3	4.5	6.0	4.5	38.3
5.0	51.6	5.0	17.3	5.0	68.9
5.5	108.7	5.5	...	...	...
6.0	262.0	5.9	126.1	6.0	388.1
6.5	616.0	6.3	...	...	...
7.0	2,135.0	6.8	757.9	6.7	2,893.0
Initial *p*H of Buffer	Triple Sulfonamide Mixt.[b] A	Final *p*H at 37°	Triple Acetyl Sulfonamide Mixt.[c] B	Final *p*H at 37°	Total Sulfonamides Columns A + B
4.5	95.9	4.5	119.0	4.5	214.4
5.0	98.4	5.0	119.0	5.0	217.4
6.0	108.9	5.8	152.7	6.0	261.6
7.0	186.0	6.9	390.2	6.8	576.2
8.0	476.0	7.5	...	...	...

[a] Equilibrium time, eighteen hours.

[b] A mixture of equal parts of sulfadiazine, sulfamerazine, and sulfamethazine, used in sufficient excess to insure saturation of each component.

[c] A mixture of equal parts of N^4-acetyl derivatives of sulfadiazine, sulfamerazine, and sulfamethazine. A sufficient excess was used to insure saturation of each component.

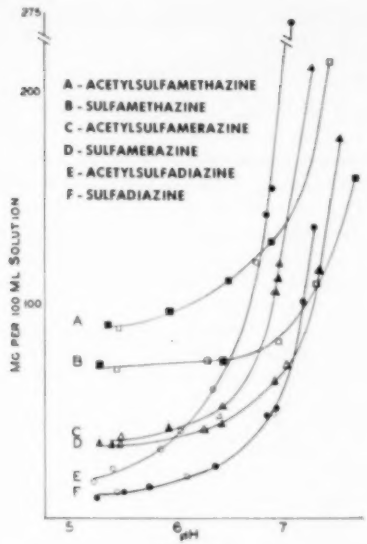

Figure 2.

Solubility of sulfapyrimidines and their N^4-acetyl derivatives.

Equilibrium time: 18 hr. at 37°. Solid symbols: phosphate buffer. Open symbols: citrate buffer. Recalculated from data of Gilligan and Plummer (11).

general the acidity tends to be increased in the greater number of pathological disorders, and also in cases where the urine is concentrated from any cause, including fevers (9). The data of Flippen and Reinhold (10) show that the *p*H was 5.5 or less in 26% of over 200 urine samples obtained from patients with acute infections. They pointed out that the observed *p*H was probably higher than that of urine in the bladder, owing to loss of carbon dioxide prior to measurement. Furthermore, it is known that there is a daily fluctuation in urinary *p*H from the more acid-fasting values in the morning to the more alkaline values later in the day, so that for a certain portion of the day the prevailing urinary *p*H is usually distinctly lower than that of the normally reported twenty-four-hour sample.

All of these considerations suggest the importance of the *p*H range 5.0 to 5.5 in studying the solubilities of sulfa drugs. Surely it is in this range that the danger of renal complications is most acute, all other factors being equal. The very gradual decline in the solubility of the sulfadiazine-sulfamerazine-sulfamethazine mixture as the *p*H is reduced from 6.0 to 5.0 bespeaks a definite clinical advantage.

The inclusion of the acetyl derivatives in this consideration appears reasonable because a considerable portion of sulfonamides is excreted in the form of the acetyl derivative. The validity of the use of the total solubility of the free drugs plus the acetyl derivatives in these considerations rests upon the assumption that each sulfonamide is acetylated to such an extent that the ratio of free drug to acetyl derivative in the urine is the same as the ratio of their solubilities, which, of course, is not quantitatively correct. However, the assumption appears sufficiently close to the truth to be useful for illustrative and comparative purposes. The question of the solubility of the acetyl derivative must undoubtedly be included in any consideration of the safety of a sulfonamide.

SUMMARY

1. The total solubility of a mixture consisting of equal parts of sulfadiazine, sulfamerazine, and sulfamethazine and a similar mixture of their acetyl derivatives was determined at 37° in the range of urinary *p*H 4.5 to 7.5.

2. The solubility of N^1-(3,4-dimethyl-5-isoxazolyl) sulfanilamide and its acetyl derivative was also determined under similar conditions, and a comparison with the triple sulfonamide mixtures was made.

3. At a *p*H level of approximately 5.5 the solubility of N^1-(3,4-dimethyl-5-isoxazolyl) sulfanilamide is equal to that of the triple sulfonamide combination. At higher *p*H levels, the solubility of the N^1-(3,4-dimethyl-5-isoxazolyl) sulfanilamide exceeds that of the sulfonamide mixture. However, at *p*H levels below 5.5 the solubility of the triple sulfonamide mixture is higher than that of the N^1-(3,4-dimethyl-5-isoxazolyl) sulfanilamide compound.

4. A similar relationship exists between the solubilities of the respective N^4-acetyl derivatives, but here the point of equality is about *p*H 6.1.

5. The practical implications of these observations are briefly discussed.

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The Determination of Ethylene Glycol in Procaine Penicillin*

By C. E. BRICKER and J. K. LEE†

Since the use of ethylene oxide has been introduced as a means of sterilizing procaine penicillin, the need has existed for an analytical method to determine trace amounts of any ethylene glycol that may have been formed by hydrolysis of the ethylene oxide. A method has been developed, involving a periodate oxidation followed by a formaldehyde determination, which is quite rapid, sensitive, and specific for small amounts of ethylene glycol and ethylene oxide.

WHEN procaine penicillin is sterilized with ethylene oxide, part of the oxide may be hydrolyzed by any moisture that may be present. The resulting ethylene glycol, which is a rather toxic substance, is nonvolatile and will remain in the sample. It is essential, therefore, to have a sensitive method to determine this possible contaminant in procaine penicillin.

Ethylene glycol is usually determined by means of periodic acid. With macro quantities, the amount of periodic acid consumed by the glycol is usually determined volumetrically. For smaller quantities of this material, Warshowsky and Elving (1) removed the formaldehyde formed from the periodic acid oxidation by distillation and determined the formaldehyde polarographically.

Colon, *et al.* (2), in their method for determining dihydrostreptomycin with periodic acid, removed the formaldehyde by distillation and then analyzed the distillate with chromotropic acid. Fleury and Boisson (3) have shown that many organic compounds which should not be affected produce some formaldehyde when heated with periodic acid. Thus, if a formaldehyde determination after a periodate oxidation is to be used as an indirect method for detecting microgram quantities of ethylene glycol in procaine penicillin, it is necessary to have either a method where iodic acid and the excess periodic acid does not interfere with the formaldehyde determination, or a method for removing formaldehyde which precludes the possibility of formaldehyde being produced from the other organic material present.

Although procaine penicillin reacts slowly with periodic acid, no formaldehyde is formed when the reaction is carried out at room temperature. On the other hand, one mole of ethylene glycol when treated with excess periodic acid produces two moles of formaldehyde. Thus, a simple method to determine formaldehyde in the reaction mixture of procaine penicillin and periodic acid would provide an analytical method to determine ethylene glycol in this penicillin salt. The chromotropic acid method (4), as well as the method investigated by Tanenbaum and Bricker (5) for determining formaldehyde, shows positive interference when strong oxidizing agents such as iodic and periodic acids are present. This interference can be eliminated by precipitating these acids with lead acetate. Although the formaldehyde can be determined on the filtrate by either method, only the chromotropic acid method will be given in the subsequent procedure

EXPERIMENTAL

Reagents

(*a*) Periodic acid, freshly prepared aqueous solution: 50 mg. of H_5IO_6 per ml.

(*b*) Lead acetate, aqueous solution: 150 mg. of $Pb(OOCCH_3)_2.3H_2O$ per ml.

(*c*) Ethylene glycol, very dilute aqueous solution containing a known quantity (e. g., 50 μg.) per ml.

(*d*) Chromotropic acid, aqueous solution of 1,8-dihydroxynaphthalene-3,6-disulfonic acid: 100 mg. per ml.

(*e*) Sulfuric acid, concentrated.

PROCEDURE

Collect two samples of procaine penicillin—one before the treatment with ethylene oxide, to be used as a blank, and the other after the treatment. Weigh into separate 10-ml. volumetric flasks one 0.50-Gm. sample of the ethylene glycol-free procaine penicillin and two 0.50-Gm. samples of the material to be analyzed for the glycol. Add 5 ml. of water to the blank and 5 ml. of water to one of the samples. To the other sample, add a known quantity of the ethylene glycol solution and sufficient water to make a total of 5 ml. Add 1.0 ml. of the periodic acid solution to each flask and swirl occasionally. After ten minutes (± one-half minute), add 1.0 ml. of lead acetate solution and dilute each solution to 10 ml. Centrifuge each mixture for five minutes at about 1,600 r. p. m. Pipette 1.0-ml. aliquots of each of the centrifugates into test tubes; add 0.5 ml. of chromotropic acid solution and 6.0 ml. of concentrated sulfuric acid. Heat the solutions in a

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boiling-water bath for thirty minutes (± three minutes). Cool under tap water and dilute each solution to 25 ml. in a volumetric flask. Allow the diluted solutions to reach room temperature and then measure the optical density of the two samples against the blank at 570 mμ.

The amount of ethylene glycol in the unknown procaine penicillin can be calculated from the following equation:

$$\mu g. \text{ of ethylene glycol in unknown} = \frac{D_1}{\frac{D_2 - D_1}{x}}$$

where

D_1 = optical density of unspiked sample.
D_2 = optical density of spiked sample.
x = Micrograms of ethylene glycol added to the spiked sample.

DISCUSSION

Procaine penicillin reacts slowly with periodic acid to produce a yellow solution. This color becomes deeper the longer the penicillin remains in contact with the oxidizing agent. Even though the yellow color does not interfere with the method for the formaldehyde determination, it is advantageous to keep the reaction time with the periodate to a minimum. It has been found that very small amounts of ethylene glycol react rapidly and quantitatively with periodic acid. When 150-μg. samples of ethylene glycol in 5 ml. of solution were treated with 50 mg. of periodic acid, only 87% of the theoretical yield of formaldehyde was obtained with one-minute reaction times. However, if the reaction mixture was allowed to stand three minutes or more before adding the lead acetate, theoretical yields of formaldehyde were obtained. Thus, the suggested reaction time of ten minutes in the recommended procedure is quite adequate to give good yields of formaldehyde from the glycol and causes no detectable interference from the reaction with the procaine penicillin.

When quantities of ethylene glycol over the range of 6 to 500 μg. are run through the recommended procedure, quantitative yields of formaldehyde are always found when calculated from a pure formaldehyde calibration curve. However, when 0.5 Gm. of procaine penicillin is added, the amount of formaldehyde found is not stoichiometric but always a constant percentage of the theoretical amount. Thus, a linear calibration curve for the determination of ethylene glycol in the presence of 0.5-Gm. quantities of procaine penicillin can be constructed and is shown in Fig. 1. It is possible, therefore, to eliminate the spiked sample from the recommended procedure and merely calculate the ethylene glycol in the unknown sample from its measured optical density and the calibration curve. However, it was found that not all samples of procaine penicillin give exactly the same calibration curve and since it is not practical to determine such a curve at frequent intervals, the most reliable method for accurate analyses is that suggested in the recommended procedure.

It is necessary to carry the blank sample of procaine penicillin through the recommended procedure because the optical densities of various lots of this material, when measured against a reagent blank, are not constant. This variation in the different samples that were used in this study could amount to about 20 p. p. m. of ethylene glycol if only a reagent blank were used instead of the procaine penicillin blank. If, however, an accuracy of 10–20 p. p. m. of the true ethylene glycol content is adequate, the blank sample of penicillin salt can usually be omitted.

The formaldehyde content of the centrifugates apparently is not stable indefinitely. For the most accurate work, the analyses of the centrifugates should be started within forty-five minutes from the time the lead acetate is added.

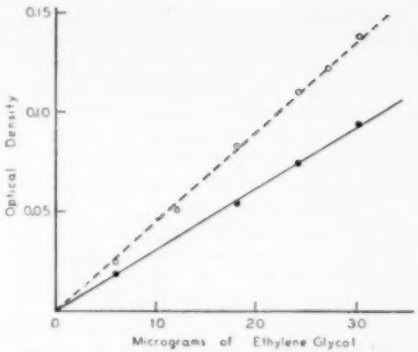

Fig. 1. Calibration curves for ethylene glycol. Open circles and dashed line, without procaine penicillin present; closed circles and solid line, with 0.50 Gm. of procaine penicillin present.

RESULTS

The validity and accuracy of this method were determined by spiking 0.5-Gm. samples from several lots of procaine penicillin with known quanitities of ethylene glycol and then analyzing them by the recommended procedure. The results of these analyses are given in Table I and show an average deviation of ±7.8 p. p. m.

TABLE I.—RECOVERY OF KNOWN QUANTITIES OF ETHYLENE GLYCOL FROM PROCAINE PENICILLIN

Ethylene Glycol Added, μg.	Ethylene Glycol Added, P. P. M.	Ethylene Glycol Found, P. P. M.	Error, P. P. M.
9	18	11	− 7
10	20	17	− 3
15	30	40	+10
18	36	42	+ 6
20	40	39	− 1
24	48	48	± 0
27	54	52	− 2
30	60	61	+ 1
40	80	89	+ 9
60	120	123	+ 3
90	180	180	± 0
120	240	221	−19
150	300	340	+40

This is thought to be exceptionally good for the analysis of trace quantities of one organic compound in the presence of other organic materials.

Four different lots of procaine penicillin which had been previously treated with ethylene oxide were analyzed on different days by several analysts. The results of these analyses are shown in Table II, and indicate that the reproducibility of the method is quite good.

If the procaine penicillin sample should contain small amounts of ethylene oxide in addition to the ethylene glycol, the oxide will be hydrolyzed during the periodic acid treatment and be determined along with the glycol. Thus this method actually determines both of these compounds and cannot distinguish between them

TABLE II.—REPRODUCIBILITY OF THE ETHYLENE GLYCOL ANALYSIS IN PROCAINE PENICILLIN

Ethylene Glycol Found, P. P. M.				
First Day	Second Day	Third Day	Fourth Day	Average
1.2	1.1	2.7	1.0	1.5
39.0	35.0	42.0	29.0	36.0
77.0	60.0	50.0	56.0	61.0
96.0; 102.0	68.0	98.0	94.0	92.0

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The Synthesis of Some Effective Antioxidants*,†

By LI-CHIN CHIANG and OLE GISVOLD‡

A description is given of the synthesis of three tetrahydroxy biphenyls: 2,2′,3,3′-tetrahydroxy biphenyl, 2,2′,3,3′-tetrahydroxy-5,5′-diethyl biphenyl, and 2,2′,5,5′-tetrahydroxy-3,3′-dimethoxy biphenyl. These three compounds were synthesized from two dehydroguaiacol derivatives, which in turn were obtained from oxidative coupling of the corresponding guaiacols. The antioxidant properties of the tetrahydroxy biphenyls are compared with NDGA and with 2,2′,3,3′-tetrahydroxy-5,5′-dimethyl biphenyl.

THE DEVELOPMENT of antioxidants is based upon the fact that the deterioration of the lipoid content of foods, pharmaceutical preparations, and many industrial goods occurs frequently after long periods of storage in the presence of air. Several types of rancidity in fats have been listed by Lea (24). In regard to these types, efforts have been made to prevent deterioration caused by the action of enzymes and by atmospheric oxidation.

The mechanism of lipoxidase oxidation has been studied by many investigators (4, 5, 20, 21). In all the studies for lipoxidase action, the spectrophotometric assay developed by Theorell, *et al.* (34), has been used.

The mechanism of autoxidation by atmospheric oxygen has interested many scientific workers for nearly a century (9, 30). Due to the complicated nature of the glyceride oils and to inadequate analytical methods, very little was known about the changes which take place during the autoxidation.

In recent years, several groups of investigators have concentrated their studies on fatty materials of known structures and high purity. By attacking the problem in such a way, together with the use of many new techniques such as ultraviolet spectrophotometry and chromatographic analysis, etc., much information has been obtained (1, 2, 6, 25, 27, 31, 33).

Most of the effective organic inhibitors for fat oxidation are phenolic in nature (29). Other substances which reinforce or increase the effect of the phenolic compounds, but have little if any activity *per se*, are "synergists." They are mostly hydroxy acids, either organic or inorganic.

The oxidation of phenolic antioxidants has been suggested to be a two-step reaction (10) and semiquinone has been proposed as an intermediate.

The mechanism of the synergism was studied by Golumbic (19). On the basis of oxidation-

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reduction potentials, he proposed that the antioxidant oxidized by fat peroxide receives hydrogen from the synergist, and in turn the reduced antioxidant transfers hydrogen to other fat peroxides and breaks the chain reaction again.

Calkins (7) further proposed that the combination of oxidized antioxidant and synergist renders the latter fat soluble.

The synthesis of biphenols with either a catechol or hydroquinone structure is stimulated by the fact that several α, ω-bis-(3,4-dihydroxyphenyl) alkanes (18), especially NDGA (26), are effective antioxidants. Other biphenyls such as 2-hyhydroxy, 4-hydroxy and 4,4′-dihydroxy biphenyls have been patented as antioxidants (22). A series of 2,2′,3,3′-tetrahydroxy-5,5′-dialkyl biphenyls with the side chains containing methyl, *n*-propyl, and *n*-amyl groups have been synthesized by Asano (3). The antioxidant properties of the methyl compound in lard, corn oil, and soybean oil showed it to be more effective than those of NDGA.

In the synthesis of biphenyls, various agents for oxidative coupling have been studied. Ferric chloride has been used by several workers on different phenolic compounds (12, 13, 15, 35). Elbs, *et al.* (16) conducted the dehydrogenation by alkali persulfate. Enzymatic oxidation and electrochemical oxidation were also applied.

EXPERIMENTAL

Dehydrodivanillin or 2,2′-Dihydroxy-3,3′-dimethoxy-5,5′-dialdoxy Biphenyl.—Dehydrodivanillin was prepared according to the Elbs, *et al.* method (16), with slight modifications in the purification (3). Colorless needles were obtained by recrystallization from butyl cellosolve.

Dehydrodiprotocatechuic Acid or 2,2′3,3′-Tetrahydroxy-5,5′-dicarboxy Biphenyl.—Dehydrodiprotocatechuic acid was prepared according to the procedure of Tiemann (3, 35). The product decomposed at 307–310° and was sparingly soluble in the common polar and nonpolar solvents.

Dehydrodicatechol or 2,2′3,3′-Tetrahydroxy Biphenyl.—Five grams of dry dehydrodiprotocatechuic acid was mixed thoroughly with 5 Gm. of Hyflo-Super Cel. The mixture was sublimed in the presence of CO_2 in a porcelain dish covered with an inverted long-neck funnel. At about 205–210°, colorless needles were collected on the wall of the funnel. The crystals turned yellow or pink if the temperature was raised above 220°. The compound decomposed at 220–222°. The whole process could be carried out in a vacuum sublimator to give a higher yield.

Dehydrodicatechol decomposed in most of the organic solvents. Recrystallization from any solvent or mixture of solvents resulted in an amorphous, pinkish solid chloride in alcoholic solution.

Anal.—Calcd. for $C_{12}H_{10}O_4$: C, 66.02; H, 4.62. Found: C, 66.59; H, 4.88.

The tetra-acetate was prepared by refluxing the phenol with acetic anhydride and powdered fused sodium acetate; m. p. 158–160°.

Anal.—Calcd. for $C_{20}H_{18}O_8$: C, 62.16; H, 4.69. Found: C, 62.60; H, 5.04.

Acetovanillone from Fries Rearrangement.—Acetovanillone was prepared from guaiacol acetate according to the procedure of Coulthard, *et al.* (11). The yield was about 50% of the theoretical, m. p. 112–115°.

Dehydrodiacetovanillone or 2,2′-Dihydroxy-3,3′-dimethoxy-5,5′-diacetyl Biphenyl.—Dehydrodiacetovanillone was obtained by the same procedure used in the preparation of dehydrodivanillin. Since acetovanillone was less soluble in water, it was dissolved in boiling water over a free flame. A gray product which decomposed at about 300° was obtained in approximately 70% yield. The compound was sparingly soluble in most of the common organic solvents.

Dehydrodi-(4-ethyl guaicol) or 2,2′-Dihydroxy-3,3′-dimethoxy-5,5′-diethyl Biphenyl by Clemmensen's Reduction.—Zinc amalgam was prepared according to the procedure of Martin (28). Before amalgamation, the mossy zinc was washed with dilute hydrochloric acid (1:1) to dissolve the coated oxides.

Ten grams of dehydrodiacetovanillone, 125 cc. of concentrated hydrochloric acid, 125 cc. of 95% alcohol, 10 Gm. of amalgamated mossy zinc, and 75 cc. of water were heated under reflux on a steam bath for an hour. Portions of 10 Gm. of amalgamated mossy zinc were added continuously at one-hour intervals until complete solution was effected. The time required was usually eight hours. The mixture was filtered while hot to remove the excess amalgamated zinc. Upon cooling in the refrigerator, a light pink precipitate was obtained and was collected by filtration. About 5 Gm. of crude product which decomposed between 134–140° was obtained. The product was purified several times by fractional solution in dilute alcohol, m. p. 140–142°.

Anal.—Calcd. for $C_{18}H_{22}O_4$: C, 71.47; H, 7.33. Found: C, 71.40; H, 7.12.

Demethylation by Hydrogen Iodide.—Three grams of dehydrodi-(4-ethyl guaiacol), 10 cc. of glacial acetic acid, 5 cc. of acetic anhydride, and 15 cc. of 47% hydrogen iodide were refluxed for two hours. The mixture was allowed to cool and was poured into 100 cc. of ice water. After standing overnight in the refrigerator, the gray residue was filtered out in about 20% yield. The gray residue was recrystallized several times from dilute alcohol containing a pinch of sodium hydrosulfite while a stream of carbon dioxide was passed to the solution. The light pink micro-crystals that were obtained decomposed at 182–185° and turned darker upon standing, even in a sealed ampul filled with CO_2.

Demethylation by Pyridinium Chloride (Pyridine Hydrochloride).—Pyridinium chloride was prepared by passing anhydrous hydrogen chloride into dried pyridine or by mixing calculated equal moles of pyridine and concentrated hydrochloric acid. The mixture was distilled and the portion boiling at 218–219° was collected.

The demethylation was carried out according to Erdtman (17) with slight modifications in the purification.

The ethereal extract of dehydrodi-(4-ethyl catechol) was dried with anhydrous Na_2SO_4 and $NaHSO_3$ and was filtered through an Al_2O_3 column in an atmosphere of N_2 in the absence of moisture. The light brown ethereal solution was evaporated upon a steam bath by blowing N_2 upon it. Light gray micro-crystals which decomposed at 182–185° were obtained in about 50% yield. (The process of crystallization must be carried out very carefully, otherwise the product will be pinkish purple in color.)

Anal.—Calcd. for $C_{16}H_{18}O_4$: C, 70.05; H, 6.62. Found: C, 70.18; H, 6.86.

The tetra-acetate was prepared by reductive acetylation. One-half gram of dehydrodi-(4-ethyl catechol) was dissolved in 10 cc. of acetic anhydride under refluxing. Three additions of 1 cc. of glacial acetic acid and 1 Gm. of zinc dust at ten-minute intervals were followed by the addition of 0.5 Gm. of fused sodium acetate and the mixture was refluxed for forty minutes. The filtrate from the zinc dust was poured into 50 cc. of ice water. The slightly gray micro-crystals obtained were recrystallized from alcohol several times. A product with a melting range of 90–94° was obtained in about 80% yield.

Anal.—Calcd. for $C_{24}H_{26}O_4$: C, 65.13; H, 5.93. Found: C, 65.08; H, 5.77.

Dehydrodi-(2-methoxy hydroquinone) or 2,2′,5,-5′-Tetrahydroxy-3,3′-dimethoxy Biphenyl. Dehydrodi-(2-methyoxy-hydroquinone) was prepared according to Dakin's reaction (14). The ethereal extract of dehydrodi-(2-methoxy hydroquinone) was dried, filtered, and concentrated. The reddish-brown, syrupy liquid solidified to a dark crystalline mass after standing. This crystalline mass was washed with ethylene dichloride in a Soxhlet extractor. The more-or-less decolorized compound was dissolved in a mixture of alcohol and benzene (1:1). The filtrate was concentrated in a vacuum desiccator until it began to crystallize. A large amount of ethylene dichloride was added while stirring. The almost white microcrystals decomposed at 170–172°. (The product decomposes in most of the organic solvents, therefore the process of crystallization must be carried out very carefully.)

Anal.—Calcd. for $C_{14}H_{14}O_6$: C, 60.41; H, 5.07. Found: C, 59.07; H, 5.27.

The tetra-acetate was prepared by the same procedure as used in the preparation of the tetra-acetate of dehydrodicatechol. Colorless crystals which melted at 179–182° were obtained in about 80% yield.

Anal.—Calcd. for $C_{22}H_{22}O_{10}$: C, 59.17; H, 4.97. Found: C, 59.26; H, 5.09.

Ferric Chloride Color Tests.—(*a*) Alcoholic solution of dehydrodicatechol gave a purplish-blue coloration after the addition of 2% $FeCl_3$.

(*b*) Alcoholic solution of dehydrodi-(4-ethyl catechol) gave a black coloration after the addition of 2% $FeCl_3$.

(*c*) Alcoholic solution of dehydrodi-(2-methoxy-hydroquinone) gave an orange-brown coloration after the addition of 2% $FeCl_3$.

Antioxidant Test.—The antioxidant properties of the three tetrahydroxy biphenyls were tested, with and without the synergist citric acid, in lard,[1] corn oil,[2] and soybean oil,[3] NDGA and dehydrodi-(4-methyl catechol) being taken as the standard for comparison. The method employed was the Swift Stability Test at 97–100° (23), using both the peroxide content (36) and methyl red indicator (8, 32) for determining the end point of the induction period. An oil bath was used (3, 8) instead of the steam bath. Results are shown in Table I.

The results were expressed in terms of the antioxidant index, i. e., the ratio of the keeping time of fat with antioxidant to that of the unprotected fat.

TABLE I.—PROTECTIVE INDICES IN LARD, CORN OIL AND SOYBEAN OIL

Antioxidant Added	Corn Oil	Soybean Oil	Lard
0.005% NDGA[a]	1.14	1.39	3.38
0.005% NDGA + 0.005% citric acid	2.33	2.34	7.81
0.005% TDMB[b]	1.79	1.41	6.82
0.005% TDMB + 0.005% citric acid	2.41	2.93	9.06
0.005% TDEB[c]	1.17	1.50	3.89
0.005% TDEB + 0.005% citric acid	2.94	2.42	6.05
0.005% THBP[d]	1.53	1.50	4.63
0.005% THBP + 0.005% citric acid	1.87	2.67	5.91
0.005% TDMO[e]	1.29	2.15	1.30
0.005% TDMO + 0.005% citric acid	2.42	2.48	19.23
0.01% TDMO	...	...	1.72
0.01% TDEB	1.60	1.73	...
0.01% TDEB + 0.005% citric acid	2.77	2.68	...
0.01% THBP	1.61	1.89	...
0.01% THBP + 0.005% citric acid	1.90	2.35	...
0.005% citric acid	1.58	1.32	1.25

[a] NDGA = nordihydroguaiaretic acid.
[b] TDBM = 2,2′,3,3′-tetrahydroxy-5,5′-dimethyl biphenyl.
[c] TDEB = 2,2′,3,3′-tetrahydroxy-5,5′-diethyl biphenyl.
[d] THBP = 2,2′,3,3′-tetrahydroxy-biphenyl.
[e] TDMO = 2,2′,5,5′-tetrahydroxy-3,3′-dimethoxy biphenyl.

SUMMARY

1. Three new tetrahydroxy biphenyls were prepared and their antioxidant properties were tested by the Swift Stability Method. Their activities in lard, corn oil, and soybean oil in 0.005 per cent concentration with or without synergist citric acid were comparable to NDGA.

2. 2,2′,3,3′-Tetrahydroxy biphenyl was prepared from vanillin via oxidative coupling by persulfate to give dehydrodivanillin, followed by subsequent fusion with potassium hydroxide to yield dehydrodiprotocatechuic acid which was decarboxylated by heating.

3. 2,2′,3,3′-Tetrahydroxy-5,5′-diethyl biphenyl was prepared from acetovanillone via oixdative coupling by persulfate to give dehydrodiacetovanillone, followed by Clemmensen reduction and subsequent demethylation.

[1] Kindly supplied by the Armour Packing Co. of St. Paul, Minn.

[2] Kindly supplied by the Corn Products Refining Co., Argo, Ill.

[3] Kindly supplied by the A. E. Staley Manufacturing Co., Decatur, Ill.

4. 2,2′,5,5′ Tetrahydroxy-3,3′-dimethoxy biphenyl was prepared from dehydrodivanillin via Dakin's reaction.

5. The antioxidant properties of the three tetrahydroxy biphenyls in 0.005 per cent concentration were found to be less active than 2,2′-3,3′-tetrahydroxy-5,5′-dimethyl biphenyl in the same concentration in lard and corn oil.

6. In lard, very promising results were obtained with 0.005 per cent 2,2′,5,5′-tetrahydroxy-3,3′-dimethoxy biphenyl using 0.005 per cent citric acid as the synergist. It was about 2.5 times more active than NDGA with citric acid and 2.1 times more active than 2,2′,3,3′-tetrahydroxy-5,5′-dimethyl biphenyl with citric acid, all in the same concentration.

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A Study on Two Colorimetric Assays for *Solanaceae* Alkaloids*

By A. B. COLBY† and J. L. BEAL‡

Procedures for two colorimetric assays for *Solanaceae* alkaloids are described. A comparison of the two colorimetric assays with the official assay of belladonna and stramonium is made. The merits of the assays are pointed out.

IN CARRYING OUT a research problem on *Solanaceae* involving numerous assays, it was found that a rapid assay, sufficiently accurate to give at least comparative results and sensitive enough to assay small amounts, was needed.

The official assay (1, 2) requires 10 Gm. of material; it is also time-consuming, requiring a minimum of three hours for extraction in addition to the assay determination. Assaying 50 to 60 samples, using the official assay procedure, would be a very time-consuming undertaking.

Two colorimetric assays have previously been reported (3–6) which show possibilities of having the above requirements. The first is an assay (3, 4) based on the purple color formed in performing Vitali's color test for *Solanaceae* alkaloids. The second assay (1, 5, 6) involves the use of ammonium reineckate to precipitate the alkaloids as alkaloidal reineckates, which are then dissolved in acetone to form a colored solution.

The Vitali colorimetric assay has been altered in such a way that the color may be measured on an electrophotometer. The reineckate colorimetric assay, which was previously used for the assay of tablets and liquid proprietaries, has been adapted to the assay of a crude drug. This paper

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is to report the comparison of results of these two colorimetric assays with the official assay for belladonna and stramonium leaf.

EXPERIMENTAL

Vitali Colorimetric Assay

Reagents.—Ethyl alcohol, 95%; ammonium hydroxide solution, 10%; chloroform; acetic acid, 6%, made with 5% alcohol; fuming nitric acid; acetone; methyl alcoholic potassium hydroxide solution, 3%.

Procedure.—Evenly wet 1 Gm. of powdered drug with 1 cc. of ethyl alcohol and 0.1 cc. of 10% ammonium hydroxide solution. Add about 5 cc. of chloroform and heat the mixture to boiling for two or three minutes. Transfer the material to a miniature percolator, previously plugged with a small wad of chloroform-soaked cotton and suspended within a stoppered, graduated cylinder of 100-cc. capacity. Percolate the drug with warm chloroform at a rate of approximately one drop per second until a volume of 31 cc. of percolate is obtained. To the percolate add sufficient 6% acetic acid to bring the level of the liquid to 80 cc. Stopper the cylinder and invert slowly about 50 times, then allow to stand until the immiscible layers separate. Remove 5–10 cc. of the acid (upper) layer by means of a pipette and pass through a dry filter paper. Transfer an exact 1-cc. aliquot of the filtrate to an evaporating dish and evaporate to dryness on a water bath. Add immediately 0.2 cc. of fuming nitric acid, making sure that the acid makes contact with the whole of the alkaloidal residue. Evaporate the mixture to dryness. Dissolve the residue in acetone and transfer quantitatively to a 25-ml. volumetric flask. Allow the contents to cool, and adjust the volume to 25 cc. with acetone. A purple coloration is produced by the addition of 0.1 cc. of 3% potassium hydroxide solution.

Determine the per cent transmittancy of the colored solution at exactly seven minutes from the time color is first produced, using a Ceneo-Sheard electrophotometer with a green filter and using acetone for the blank. The reading obtained is correlated with the amount of alkaloid present in the 1-cc. portion assayed by referring to a standard curve.

If 1 cc. is the volume assayed, then the calculation is:

$$\% \text{ alkaloid} = \frac{\text{amount of alkaloid in 1 cc.} \times 50}{\text{weight of sample}} \times 100$$

Preparation of Standard Curve.—The standard curve was obtained by dissolving a known amount of hyoscyamine sulfate in chloroform and assaying appropriate aliquot portions as in the above procedure for assaying the percolate. Appropriate corrections must be made so that the values correspond to hyoscyamine and not hyoscyamine sulfate. Plot the per cent transmittancy on semilogarithmic graph paper, 1 cycle × 12 divisions per inch. Values obtained and plotted in this study are given in Table I.

Additional notes concerning this colorimetric method may be obtained from the report of Allport and Wilson (3).

Samples of leaves of *Datura tatula* Linné and *Atropa belladonna* Linné grown in the Ohio State University Pharmaceutical Garden were assayed by the official method and by the Vitali colorimetric method. Results obtained are given in Table II.

From the results obtained, one observes that this colorimetric assay does not give results that are as high as the results obtained from the official assay. The average deviation from the mean is also greater for the Vitali colorimetric assay than for the official assay, thus causing the 0.95 confidence interval to be greater.

TABLE I.—VALUES FOR STANDARD CURVE OF VITALI COLORIMETRIC ASSAY

Hyoscyamine, Mg.	Transmittancy, %
0.05	77.2
0.10	61.0
0.15	51.8
0.20	41.0
0.25	34.0
0.30	27.0

Two main sources of error are: (*a*) If an error is made at any time while assaying the 1-cc. aliquot portion, by nature of the calculation the error is multiplied 50 times; and (*b*) the color formed is not stable. Thus the reading must always be taken at the same time intervals as when making the standard graph curve.

Advantages of Assay.—(*a*) The assay is very simple and can easily be completed in an hour. When the assayer is properly prepared, he may even complete two or three assays in an hour. (*b*) The color test is sensitive and a very small amount of alkaloid can be determined. The smallest amount used in standardizing the curve was 0.05 mg., but a smaller amount can be determined. (*c*) Even though the results are not as high as the official assay results, this colorimetric assay could be used for comparative purposes.

Ammonium Reineckate Colorimetric Assay

Reagents.—Ammonium reineckate aqueous solution, 1%; sulfuric acid, 0.5 *N*; ethyl alcohol, 95%; ammonium hydroxide solution, 10%; chloroform; acetone.

Preparation of Standard Curve.—Dissolve 100 mg. of hyoscyamine sulfate in 15 cc. of 0.5 *N* sulfuric acid. Make alkaline with 10% ammonium hydroxide solution and extract with chloroform. Remove aliquot portions and transfer to 50-cc. beakers. Evaporate the chloroform solution until nearly dry, add 5 cc. of 0.5 *N* sulfuric acid, and then completely evaporate the remaining chloroform. Add 10 cc. of 1% ammonium reineckate solution to precipitate the alkaloids as insoluble reineckates. Allow to stand for thirty minutes, stirring frequently to bring about complete precipitation. Filter the solution using a sintered-glass filter of medium porosity with a small amount of suction. Wash the beaker and the precipitate with three 2-cc. portions of cold water. Place a 25-cc. volumetric test tube inside a suction flask with the stem of the sintered-glass filter within the neck of the test tube. Dissolve the precipitate in acetone by passing small portions through the filter and collecting the colored filtrate. Wash the beaker which had contained the precipitate with a

TABLE II.—RESULTS OF VITALI COLORIMETRIC ASSAYS AND OFFICIAL ASSAYS OF STRAMONIUM AND BELLADONNA

Stramonium				Belladonna			
Official Assay		Colorimetric Assay		Official Assay		Colorimetric Assay	
% Alkaloid	Deviation from Mean	% Alkaloid	Deviation from Mean	% Alkaloid	Deviation from Mean	% Alkaloid	Deviation from Mean
0.764	0.0284	0.709	0.0444	0.566	0.0265	0.510	0.0198
0.756	0.0204	0.708	0.0434	0.554	0.0145	0.522	0.0318
0.744	0.0084	0.675	0.0104	0.548	0.0085	0.435	0.0552
0.683	0.0526	0.620	0.0446	0.505	0.0345	0.459	0.0312
0.702	0.0336	0.616	0.0486	0.537	0.0025	0.525	0.0348
0.765	0.0294	0.660	0.0046	0.527	0.0125	..	..
		Official	Colorimetric			Official	Colorimetric
Mean		0.7356	0.6646	Mean		0.5395	0.4902
Av. Deviation from Mean		0.0288	0.0326	Av. Deviation from Mean		0.0165	0.0346
Standard Deviation		0.035	0.0408	Standard Deviation		0.022	0.041
Standard Error		0.0143	0.0166	Standard Error		0.0089	0.0183
0.95 Confidence Interval		0.773–0.699	0.702–0.628	0.95 Confidence Interval		0.562–0.517	0.541–0.439

little acetone and add the washings to the filtrate. Add sufficient acetone to the filtrate to bring the volume to 25 cc., stopper, and thoroughly mix. Measure the transmittancy electrophotometrically. A Fisher electrophotometer, 115 volts, 60 cycles, a.-c. model with a 525B filter, and acetone as a blank was employed in this work. Values obtained for preparation of the standard curve are given in Table III.

TABLE III.—VALUES FOR STANDARD CURVE OF AMMONIUM REINECKATE ASSAY

Hyoscyamine Alkaloid, Mg.	Transmittancy, %
15.0	62.5
12.5	68.0
10.0	73.5
7.5	79.5
5.0	86.0
2.5	93.0

The readings obtained for the standardization were plotted on semilogarithmic graph paper, 1 cycle × 10 divisions per inch.

Procedure for Assay.—Evenly wet 1 Gm. of powdered drug with 1 cc. of 95% ethyl alcohol and 0.1 cc. of 10% ammonium hydroxide. Add about 5 cc. of chloroform and heat the mixture to boiling for two or three minutes. Transfer the material to a miniature percolator, previously plugged with a small wad of chloroform-soaked cotton and suspended within a 60-cc. separatory funnel. Percolate the drug with warm chloroform at a rate of approximately one drop per second until at least 30 cc. is collected in the separatory funnel. Extract the chloroform solution with five successive 10-cc. portions of 0.5 *N* sulfuric acid. Make the solution alkaline by adding 10% ammonium hydroxide solution and completely extract it with chloroform. The chloroform solution is then treated in the same manner as used in the preparation of the standard curve.

Samples of leaves of *Atropa belladonna* Linné, grown in the Ohio State University Pharmaceutical Garden, were assayed by the official method and by the ammonium reineckate assay. These samples were not those used in the Vitali colorimetric assay. Results obtained are given in Table IV.

Notes on the Method.—(*a*) Atropine sulfate gives results identical with those for hyoscyamine sulfate. (*b*) The amount of 5 cc. of 0.5 *N* sulfuric acid added just before the addition of the ammonium reineckate solution is not critical. (*c*) The thirty-minute period allowed for complete precipitation of the alkaloidal reineckate was found to be longer than necessary for the above assays, but was used as a matter of convenience. (*d*) The 10 cc. of ammonium reineckate solution added as precipitant is an excess amount, and in most cases a smaller amount could be safely used if so desired.

CONCLUSIONS

1. The reineckate colorimetric assay gives results that are more comparable to the official assay than are the results of the Vitali colorimetric assay. Standard error for the reineckate colorimetric assay was even smaller than for the official assay.

2. The color developed in the reineckate assay is much more stable than the color developed in the Vitali colorimetric assay.

3. The Vitali colorimetric assay is more sensitive than the reineckate colorimetric assay in that smaller amounts of alkaloid can be determined. Thus it can be used to assay single immature plants when comparative results are desired.

4. The Vitali colorimetric assay is more rapid than the reineckate colorimetric assay. Both as-

TABLE IV.—RESULTS OF REINECKATE COLORIMETRIC ASSAYS AND OFFICIAL ASSAYS OF BELLADONNA

Official Assay		Colorimetric Assay	
% Alkaloid	Deviation from Mean	% Alkaloid	Deviation from Mean
0.445	0.0504	0.496	0.0006
0.511	0.0156	0.523	0.0276
0.517	0.0216	0.499	0.0036
0.506	0.0106	0.456	0.0394
0.498	0.0026	0.503	0.0076
		Official	Colorimetric
Mean		0.4954	0.4954
Av. Deviation from Mean		0.0202	0.0158
Standard Deviation		0.029	0.024
Standard Error		0.0129	0.0107
0.95 Confidence Interval		0.531–0.460	0.525–0.465

says are more rapid than the official assay and require less material for the assay.

SUMMARY

A comparison of two colorimetric assays with the official assay of belladonna and stramonium was made. One colorimetric assay was based on the Vitali color reaction; the other colorimetric assay was based on an alkaloidal reineckate precipitate which gives a color when dissolved in acetone. Both assays were found to be rapid and simple to perform. The reineckate assay was the more accurate, whereas the Vitali assay was more sensitive and more rapid. The Vitali assay is accurate enough to show comparative results when only a small amount of material to be assayed is at hand.

A research project is now in progress in which a comparison of these methods on a larger scale is being undertaken.

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The Separation of the Ergot Alkaloids by Paper Partition Chromatography*,†

By V. E. TYLER, Jr. and A. E. SCHWARTING

The problem of separation of ergot alkaloids in solvent systems is complicated by the varied solubility of the alkaloids and by their instability. The conventional procedure of paper partition chromatography, in which water is the stationary phase, does not separate the ergotoxine and ergotamine alkaloids since they follow the solvent front of the wash liquid. A partition coefficient, favoring the stationary phase, was achieved by substituting an organic-aqueous system for the water. The stability of the alkaloids was assured by using acidic wash liquids.

IN THE COURSE of the investigation of the biosynthesis of ergot alkaloids by *Claviceps purpurea* in submerged culture, it became necessary to be able to detect minute quantities of material. This objective, together with a method for the separation of all the alkaloids, has been achieved by the process of paper partition chromatography.

Ergonovine and ergometrinine have been separated by paper partition chromatography (1). A recent note (2) indicated a method for the separation of the water-insoluble alkaloids on buffered filter paper. A detailed report of this investigation has appeared (3). The impregnation of filter paper with glycols (4) and procedures for reverse phase partition chromatography have been described (5–7).

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MATERIALS

Alkaloids.—Ergotoxine ethanesulfonate B. P., ergonovine maleate U. S. P., and ergotamine tartrate U. S. P. were used in the preparation of the alkaloids. Ergotoxine and ergotamine were each prepared by dissolving 100 mg. of each salt in 25 cc. of water and 5 cc. of acetone. Sodium bicarbonate was added to saturation. The base was collected, washed with water, and dried over phosphorous pentoxide. Ergonovine was prepared by dissolving 100 mg. of the maleate in 2 cc. of ethanol and 3 cc. of stronger ammonia T. S. Fifty cubic centimeters of an aqueous, saturated sodium chloride solution was added. The base was extracted with ether. The solution was dried and the solvent was evaporated. The product was dried over phosphorous pentoxide.

Ergometrinine was prepared by dissolving 25 mg. of the base in 0.3 cc. of glacial acetic acid. Three cubic centimeters of water was added and the mixture was refluxed for one hour. The solution, made alkaline with ammonium hydroxide, was extracted with ether. The residue after evaporation was collected. Ergotinine and ergotaminine were each prepared by refluxing 25 mg. of the l-base of each compound in

2 cc. of methanol for one hour. The reaction products, each containing the isomeric pairs, were dissolved in ethanol for use.

Lysergic acid, equivalent to 128.6% of ergonovine maleate by colorimetric assay, was used.

The fluorescence of the alkaloids served as a means for their localization on paper. The zones of distribution of all alkaloids were detectable when 5 μg. was chromatographed, although the water-insoluble alkaloids are more readily detectable when 10–20 μg. are used, since the wash liquids used in this investigation caused a damping of their fluorescence.

The levorotatory alkaloids and their salts were used in ethanol solutions containing 5 μg./20 μl. Twenty-microliter volumes were chromatographed. Forty microliters each of the ergonovine-ergometrinine mixture in ether and the ergotoxine-ergotinine and ergotaminine mixtures in ethanol were used. Each volume of the isomeric pairs represented approximately 10 μg. of alkaloids. Lysergic acid was applied in 20-μl. volumes containing 5 μg. of the substance.

Wash Liquids.—A *n*-butanol-glacial acetic acid-water (4:1:5) mixture was prepared. The saturated butanol layer and the saturated water layer were used as wash liquids in separate experiments. The aqueous layer had a *p*H of 2.1.

Phosphate-citric acid buffer systems (8), 6 volumes, at *p*H levels of 3.0, 4.5, and 6.0 were each equilibrated with 4 volumes of *n*-butanol. The aqueous phase of each was used as a wash liquid.

EXPERIMENTAL

The procedure of paper partition chromatography employing the descending method was used in all procedures except those reported in Table I. Strips of Whatman No. 1 paper, 1 x 22½ in., or sheets, 18¼ x 22½ in. were used. The solvent front was allowed to move at least three-fourths of the length of the paper at a temperature of 21° ± 1.0°. The R_F values of the levorotatory alkaloids and their common salts and of lysergic acid were first determined by chromatographing, by ascending technique, on strips. The wash liquid, butanol-acetic acid-water (butanol phase), gave values reported in Table I.

This method did not give separation of the water-insoluble alkaloids, but provides a method for the detection of lysergic acid and ergonovine.

Filter paper strips were impregnated with propylene glycol, and formamide, and after application of the alkaloids, the wash liquids of benzene, tolulene, and xylene, each equilibrated with the stationary-phase liquid, were used to form the chromatograms. Although separations of the water-insoluble alkaloids were possible, the method was not adaptable to an unambiguous separation of all of the alkaloids. The combination of propylene glycol and xylene or toluene gave best results. The results of this work are not reported.

Hydrophobic filter paper was prepared by dipping strips or sheets of paper in a 5% (v/v) solution of Dow-Corning Silicone No. 1107 in heptane (Eastman, practical grade). The paper, allowed to dry in air, was heated in an oven at 150° for three minutes, and after application of the alkaloids or alkaloid mixture, was equilibrated for twelve hours with the vapor of the saturated butanol phase of the wash liquids. The chromatograms were formed with the saturated aqueous phase of the several wash liquids. The aqueous phase of the butanol-acetic acid-water system and the aqueous phase of the butanol-*p*H 3.0 buffer gave the broader separations. R_F values of the alkaloids obtained with these two systems are reported in Table II. These values must be considered relative, since it was observed that exact reproduction of the R_F values is influenced by changes in the silicone content and by differences in the curing process.

TABLE I

Compound	R_F
Ergotamine	0.98
Ergotoxine	0.98
Ergonovine	0.67
Ergotamine tartrate	0.98
Ergotoxine ethanesulfonate	0.98
Ergonovine maleate	0.66
Lysergic acid	0.51

TABLE II

Compound	R_F Values: Butanol-Acetic Acid-Water	R_F Values: Butanol-Buffer (*p*H 3.0)
Ergonovine	0.62	0.52
Ergometrinine	0.78	0.72
Ergotoxine	0.85	0.67
Ergotinine	0.87	0.68
Ergotamine	0.75	0.54
Ergotaminine	0.57	0.72
Lysergic acid	0.52	0.34
Ergonovine maleate	0.64	0.50
Ergotoxine ethanesulfonate	0.86	0.67
Ergotamine tartrate	0.78	0.54

The methods fail in the separation of ergotoxine and its isomer. This pair, ergotoxine-ergotinine, is separable when chromatographed with the butanol *p*H 4.5 buffer system. By this procedure, the R_F of ergotoxine is 0.60 and the R_F of ergotinine is 0.69.

The alkaloid bases do not tail but assume rather large areas by these processes. The wash liquids do not dissolve paper constituents, and it was noted that extracts of ergot chromatographed with remarkable clarity.

Two-dimensional chromatograms of a mixture of the levorotatory alkaloid bases and lysergic acid have been prepared. The R_F values obtained on strips are duplicated on silicone-treated sheets when the first wash is the aqueous phase of the butanol-acetic acid-water system. Equilibration of the paper with the butanol phase is necessary before each formation.

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A Pharmacognostical Study of Roots of Different Species of *Veratrum**

By HEBER W. YOUNGKEN

Studies made on roots of botanically authenticated plants of *Veratrum viride, Veratrum album, Veratrum Woodii, Veratrum californicum, Veratrum Eschscholtzii, Veratrum fimbriatum,* and a *Veratrum* from Montana are reported. Descriptions are given of the epidermal cells, the aerenchyma, the starch grains, and the endodermis. The radial, polyarch fibrovascular bundle in each of these roots varied in the number of phloem and xylem patches, and ranges are given for the different species. Thirty-two somatic chromosomes were observed in stained smears of root tips of both *Veratrum viride* and *V. album.*

FOLLOWING intensive studies during the past two or three years on the chemistry and pharmacology of *Veratrum viride* rhizomes and roots, during which period the crude drug was submitted to chemical fractionation by Fried, White, and Wintersteiner (1) resulting in the isolation of the pure crystalline ester alkaloids germidine and germitrine and, following the published results of the investigations of Fries, Stanton, and Moister (2) on the actions of the fractions and pure alkaloids in hypertensive patients which showed that germidine and germitrine exhibited high hypotensive activity in man, interest in this drug has been revived. Today, its preparations are extensively used in the treatment of hypertension and pre-puerperal eclampsia.

The demand by pharmaceutical manufacturers for the crude drug has become so great that collectors have offered a number of substitutes, samples of which have been submitted to the author for identification. The substitutes identified were the roots of *Veratrum album, V. Eschscholtzii, V. Woodii, V. californicum,* and a *Veratrum* sp. from Montana.

The purpose of this paper is to discuss the results of studies made on the anatomy of the roots of *Veratrum viride* and allied species of *Veratrum*, all but one of which, *V. fimbriatum*, have been offered as substitutes for *Veratrum viride*, and, to report the results of studies made on the number of chromosomes of *Veratrum viride* and *V. album.*

The literature dealing with the anatomy of the roots of *Veratrum* species has been concerned mostly with *Veratrum viride* and *V. album*, and to a lesser extent with *V. californicum* and *V. nigrum.* Holm (3) studied *Veratrum viride;* Meyer (4), *V. album;* Bastin (5), Schrenck (6), Denniston (7), and Flückiger (8) studied *V. viride* and *V. album.* Viehoever, Keenan, and Clevenger (9) reported on the histological characteristics of *V. viride, V. album,* and *V. californicum.*

Since the rhizomes and roots of additional species have recently been offered as substitutes for *V. viride*, the problem of control of *Veratrum viride* has become more complicated, necessitating additional comparative studies of the roots of this with other American species of *Veratrum.*

MATERIALS AND METHODS

The materials used in this investigation consisted of entire plants of *Veratrum viride* Aiton gathered in the Southern Blue Ridge, Pennsylvania, New York, Maine, New Hampshire, and Quebec; *V. album*, gathered at Erlangen near Zurich, Switzerland; *V. californicum* Durand, gathered in California; *V. Woodii* Robbins ex Gray, gathered in Missouri by R. B. Livingston; *V. Eschscholtzii* A. Gray, from Juneau, Alaska; *V. fimbriatum* A. Gray, gathered by Carl Taylor in Northern California; and a *Veratrum* gathered near Missoula, Mont., by J. H. Coleman.

With the exception of *V. Eschscholtzii, V. Woodii*, and the *Veratrum* from Montana, some of the plants were transplanted to moist woodland soil in the Waltham Field Station and Medicinal Plant Garden of the Massachusetts College of Pharmacy. Numerous samples of dried rhizomes and roots of *Veratrum* species were also employed, as generously supplied by crude drug and manufacturing pharmaceutical houses. Some of these were later identified by the author as substitutes.

Some of the fresh materials were preserved in 50 per cent alcohol and glycerin for later study. Rootlets were also detached from fresh and dried plant materials and partly dehydrated in a series of alcohols, passed through xylol into xylol-paraffin, embedded in paraffin and later sectioned, fixed to slides, stained and mounted in balsam. Others were sectioned free hand in pith and in cork and temporary mounts prepared in water, phloroglucin-HCl, and lactophenol solution. The rootlets were sectioned at various levels from base to tip and the mounts examined. More than a hundred sections of different roots of each species considered were studied. Transverse sections through the regions of maturity

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Presented to the Scientific Section, A. PH. A., Buffalo meeting, August, 1951.

of the roots were of most pharmacognostic significance in distinguishing the species.

In the chromosome study of *Veratrum viride* and *V. album*, the growing plants were unearthed to expose the rootlets, segments of the root tips snipped off, placed in vials of fixing solution and carried to the laboratory where they were placed in a mixture of equal parts of 95 per cent alcohol and HCl for a minute, transferred to Thomas' Fixative Solution for five minutes and boiled in Belling's Iron-aceto-carmine stain for three minutes. The root tips were then transferred to a clean microslide, two drops of the same stain added, the root tips minced with a sharp microscopic knife, the preparation covered with a cover slip, the cover slip pressed, and the chromosomes counted.

HISTOLOGICAL STUDY OF VERATRUM ROOTS

Veratrum Viride Root

As viewed in cross-section (Fig. 1), the epidermis consists of one layer of epidermal cells, quadratic to slightly radially elongated with curved and thickened outer walls. These are more or less longitudinally elongated with rectilinear walls in surface view and about 38 μ to 114 μ in length. In mature regions the walls were lignified.

The hypodermis or outer cortex consists of collenchyma which is up to 3 rows of cells in width.

The subjacent aerenchyma tissue exhibits radially elongated air spaces surrounded by parenchyma cells, many of which are disorganized. This region of the cortex occupies up to about one-half its width. The parenchyma cells of the cortex were thin-walled, circular, larger in the outer region, smaller toward the central cylinder. Most of them contain starch grains; some scattered ones, raphides of calcium oxalate. The starch grains are single, spheroidal to oval, and from 2- to 3-compound, the individual grains up to 20 μ in diameter with a central hilum. The raphides measured were mostly up to 102 μ in length.

The endodermis, in cross sections, consists of one row of slightly lignified cells, very irregular in size, shape, and in wall thickness. In some of the sections, certain cells possessed thin walls, but most of the endodermal cells possess radial and inner walls thicker than the outer walls. The lumen is chiefly U-shaped but occasional cells were seen in many of the sections studied which possessed more or less V-shaped lumina as found in the typical endodermal cells of the root of *Veratrum album*. An occasional cell with a quadratic lumen was observed. (See Fig. 5*V*.) In longitudinal sections, these cells are elongated and exhibit beaded thickenings on two of their walls.

Beneath the endodermis there is a central cylinder consisting of a pericycle of one to two layers of thin-walled cells surrounding a polyarch, radial fibrovascular bundle, and a central pith. The radial bundle consists of from 8 to 14 phloem strands alternating with an equal number of xylem strands in the materials examined. The pith consists of thick-walled cells which were lignified in mature regions of the root.

Veratrum Album Root

The epidermis, in transverse sections, consists of one layer of epidermal cells, quadratic to slightly radially elongated with convexly curved, thickened, outer walls. In surface view the cells resembled the epidermal cells of *V. viride* root being arranged in parallel rows, but some were up to 150 μ in length. They were more or less lignified in mature regions. The hypodermis consisted of 2 to 3 rows of collenchyma cells. The aerenchyma somewhat resembled that of *Veratrum viride* root, the air spaces being radially elongated, but usually less pronounced, extending only one-fourth to one-third the width of the cortex in the mature roots. The parenchyma consisted of thin-walled, more or less circular starch-bearing cells interspersed with raphide sacs, the latter containing acicular crystals up to about 66 μ in length. The starch grains were both simple, spheroidal to oval and 2- to 3-compound, rarely up to 5-compound. The individual grains were up to 19 μ in diameter, mostly up to 10 μ.

The endodermis consists of a single row of more or less quadrangular golden yellow cells as viewed in cross section. As in *Veratrum viride*, most of these cells possessed radial and inner walls thicker than the outer walls, while occasional cells were thin-walled. The walls were lignified. The lumina of thick-walled cells varied from V- to U-shaped. (See Fig. 5*A*.)

The polyarch, radial fibrovascular bundle consists of 8 to 15 xylem and 8 to 15 phloem patches alternately arranged, with the xylem strands surrounding the phloem on its inner margin. The pith consists of thick-walled cells which exhibit lignified walls in mature regions.

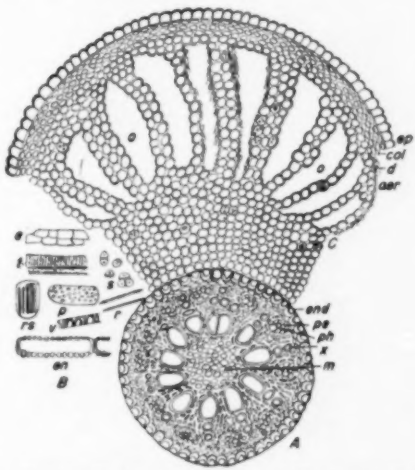

Fig. 1.—*Veratrum viride* root. *A*, Representative portion of a transverse section showing *ep*, epidermis; *col*, collenchyma of hypodermis; *aer*, aerenchyma tissue in cortex showing oxygen spaces (*o*) and disintegrated parenchyma cells; *C*, cortex; *end*, endodermis; *pe*, pericycle; *ph*, phloem; *x*, xylem of radial, polyarch fibrovascular bundle; and *m*, pith. *B*, Histological elements of powdered *Veratrum viride* root. *e*, epidermal cells in surface view; *t*, pitted vessel with adherent portions of two fibers; *s*, starch grains; *r*, raphides of calcium oxalate; *rs*, raphide sac; *p*, parenchyma cell of cortex containing starch grains; *v*, spiral vessel; *en*, endodermal cell in lengthwise view. Magnified.

Veratrum Eschscholtzii Root

The epidermis, as viewed in cross sections (Fig. 2), consists of one layer of radially elongated epidermal cells whose outer walls are convexly curved and thickened. They are nonlignified. The hypodermis in the mature region consists of 4 to 8 rows of cells, mostly collenchymatic. The aerenchyma contains radially elongated air spaces developing to about one-third the width of the cortex and are not as pronounced as in the roots of *V. viride*. The parenchyma of the cortex consists of circular, thin-walled, starch-bearing cells, interspersed among which are raphide sacs containing acicular crystals of calcium oxalate up to about 140 μ in length. The starch grains are single, spheroidal, and from 2- to 3-compound, the individual grains up to 16.5 μ and with a central hilum. Longitudinal sections exhibit long chains of raphide sacs arranged end to end in the cortex.

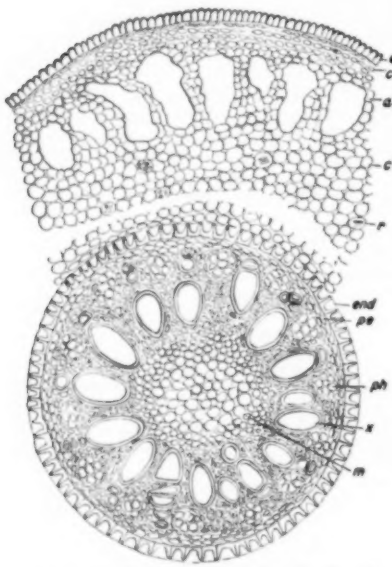

Fig. 2.—*Veratrum Eschscholtzii* root. Drawing of a representative portion of a transverse section. *ep*, epidermis; *c*, cortex; *col*, collenchyma in outer cortex; *aer*, aerenchyma; *r*, raphides of calcium oxalate; *end*, endodermis; *pe*, pericycle; *ph*, phloem; *x*, xylem; *m*, pith. Magnified.

The endodermis consists of one row of yellow to orange endodermal cells which in cross sections appear very regular in comparison with those of *V. viride* root, with an average radial length of 26.6 μ and an average tangential width of 19 μ. Their walls are nonlignified and their lumina U-shaped. (See Fig. 5*E*.) The polyarch, radial fibrovascular bundle consists of 9 to 15 alternating phloem and xylem strands. The xylem consists of pitted, reticulate, and scalariform tracheary elements and wood fibers, the latter with walls up to 15 μ in width.

The pith is composed of thick-walled, lignified cells.

Veratrum Californicum Root

The epidermis, as viewed in cross section, consists of one layer of quadratic to slightly radially elongated cells with partially lignified walls in mature regions. Their outer walls are convexly curved and thickened. In cross sections the average radial length of these cells was 43.6 μ and average tangential width, 40 μ.

The hypodermis (outer cortex) consists of 2 to 3 rows of collenchyma cells. The aerenchyma contains, in mature regions, very prominent air spaces which are radially elongated and occupy up to two-thirds the width of the cortex. Many of the cells bordering the air spaces are disorganized in mature regions of the root. The remainder of the cortex consists of more or less circular starch-bearing parenchyma cells, scattered among which are raphide sacs containing raphides of calcium oxalate, often up to 106 μ in length. The starch grains are single, spherical and 2- to 3-compound, the individual grains usually up to 16.5 μ, rarely 25 μ. The endodermis consists of one row of cells, the outer tangential walls of which are thin and the radial and inner tangential walls thickened and lignified. The lumina of these cells vary from U- to V-shape, the U-shaped type being dominant in the material examined. (See Fig. 5*C*.) The polyarch, radial fibrovascular bundle consists of from 10 to 20 alternating phloem and xylem strands. The pith consists of thick-walled, lignified cells.

Veratrum Woodii Root

The epidermis consists of one row of radially elongated epidermal cells, the average radial length of which was 25.4 μ and average tangential width, 15.5 μ.

The hypodermis consists of 3 to 4 rows of collenchyma cells.

The aerenchyma contains 1 to 2 rows of more or less diagonally elongated air spaces occupying about one-third the width of the cortex in mature regions.

The parenchyma of the remainder of the cortex consists of more or less circular thin-walled cells containing starch. Interspersed among the starch-bearing cells are raphide sacs containing raphides of calcium oxalate up to about 66 μ in length in the material examined. The starch grains are single, spheroidal to oval, and from 2- to 3-compound. The individual grains are up to about 11.5 μ in diameter.

The endodermis consists of one row of quadratic cells with more or less lignified walls. The radial and inner walls in cross sections are thicker than the outer walls. The lumina of these cells are both U- and V-shaped (see Fig. 5*W*).

The polyarch, radial fibrovascular bundle consists of 10 to 15 alternating phloem and xylem strands.

The pith consits of cells with thick lignified walls (see Fig. 3).

Veratrum Woodii roots differ from the roots of all other *Veratrum* species examined by the author in exhibiting lacunae in the pith and hypodermis.

Veratrum Fimbriatum Root

The epidermis in cross section (Fig. 4) consists of one layer of more or less quadratic cells with curved and thickened outer walls, all walls being slightly lignified in mature regions.

The hypodermis consists of 3 to 5 rows of collenchyma cells.

The aerenchyma contains very pronounced, radially elongated air spaces, extending in mature regions of roots as far as the stele, and representing probably the most diagnostic characteristic of this root.

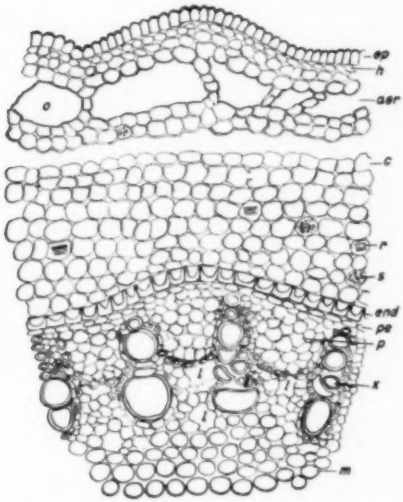

Fig. 3.—*Veratrum Woodii* root. Drawing of a representative portion of a transverse section through the mature region. *ep*, epidermis; *h*, hypodermis; *aer*, aerenchyma showing oxygen spaces (*o*); *c*, cortex showing cells with starch grains (*s*) and raphides (*r*); *end*, endodermis; *pe*, pericycle; *p*, phloem; *x*, xylem of the radial, polyarch fibrovascular bundle; *l*, lacunae; *m*, pith. Magnified.

The parenchyma consists of circular, thin-walled cells containing starch. The starch grains are single, spheroidal, and from 2- to 3-compound, the individual grains being up to 26.4 μ in diameter. No raphides of calcium oxalate were observed in the material examined. The endodermis consists of one row of quadratic cells which are rather irregular in size, and possess radial and inner tangential walls which are thicker than the outer walls. These walls are nonlignified and their thickness appeared less pronounced than in similar walls of other species of *Veratrum*.

The polyarch, radial fibrovascular bundle consists of 13 to 17 alternating phloem and xylem strands. The pith consists of thick-walled lignified cells.

Roots of a Veratrum from Montana

The dried rhizomes and roots of an unknown species or variety of *Veratrum* growing in the vicinity of Missoula, Mont., and stated to have been identified as *Veratrum viride*, together with pressed aerial portions of plants yielding the same, were received by the author from a correspondent for determination. Examination of this material showed it to differ from typical eastern *Veratrum viride*, and

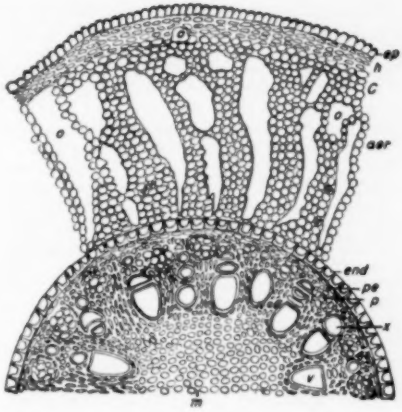

Fig. 4.—*Veratrum fimbriatum* root. Drawing of a representative portion of a transverse section, the central cylinder enlarged to about twice its proportional area to show structural details. *ep*, epidermis; *h*, hypodermis; *C*, cortex; *aer*, aerenchyma; *o*, air spaces which occur throughout the cortical regions; *end*, endodermis; *pe*, pericycle; *p*, phloem; *x*, xylem; *v*, vessel; *m*, pith. Magnified.

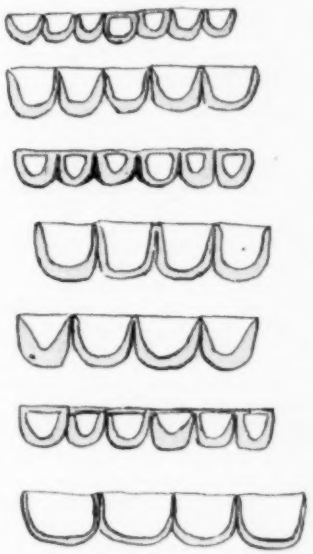

Fig. 5.—Endodermis of roots of different species of *Veratrum*. Representative portion, as viewed in transverse sections. *V*, *Veratrum viride*; *M*, *Veratrum viride*, western form from Missoula, Montana; *A*, *Veratrum album*; *E*, *Veratrum Eschscholtzii*; *C*, *Veratrum californicum*; *W*, *Veratrum Woodii*; *F*, *Veratrum fimbriatum*. Magnified.

to possess characters somewhat intermediate between *V. viride* and *V. Eschscholtzii*. A microscopical study of its root which follows revealed the presence of an endodermis (Fig. 5) somewhat different in its wall thickenings from both *V. viride* and *V. Eschscholtzii*.

The epidermis consisted of one layer of radially elongated cells with curved and thickened outer walls. The hypodermis consisted of 4 to 8 rows of collenchymatic cells. The aerenchyma possessed radially elongated air spaces up to one-third the width of the cortex in mature regions. The cortical parenchyma possessed circular, thin-walled, starch-bearing cells and scattered raphide sacs. The starch grains were single, spheroidal, and from 2- to 3-compound, the individual grains up to 16.5 μ. The raphides measured were up to 95 μ in length.

The endodermis consisted of a row of yellow, non-lignified cells averaging 22.77 x 24.01 μ whose radial and inner walls were usually more thickened than the outer walls. The lumina of these cells were mostly U-shaped. Occasional cells occurred which were nearly to quite uniformly thickened in all walls like some of the endodermal cells of *V. viride*, although most of the cells were more like those of *V. Eschscholtzii*.

The polyarch radial fibrovascular bundle possessed from 12 to 16 phloem strands and 12 to 16 xylem strands in alternate arrangement.

The pith consisted of thick-walled, lignified parenchyma in mature regions.

A comparative taxonomic study of the plants yielding this root and of some other western *Veratrums* will be necessary before the identity of this material can be established.[1]

CHROMOSOME NUMBER OF VERATRUM VIRIDE

The writer has long held the hypothesis that *Veratrum viride* and *V. album* represented ecological varieties or forms of the same species through observations of similarities between the flowers, leaves, and even the root-structure of many plants of each group. The perianth of *V. album* varies, being sometimes white but often green like that of *Veratrum viride*. Some roots of both species exhibit endodermal cells with both U- and V-shaped lumina. Both species have members with leaves similar in shape, size, and venation. A chromosome number study of *Veratrum viride* and *V. album* was undertaken in 1950 on the root tips of plants grown by the author in wet woods of the Waltham Field Station. In 1932 Stenar determined the number of somatic chromosomes in *V. album* to be 32 (10). In this study, separate observations and tests were carried out by Dr. V. H. Simonian and the writer on root tips of different *Veratrum viride* plants according to the method previously described under "Materials and Methods" in this paper. The number of 2n chromosomes found by each of us in *V. viride* was 32. The chromosome count made by Stenar for *V. album* was confirmed. A comparative study of the rhizomes of American *Veratrums* and European *V. album* is contemplated.

SUMMARY

1. A report is given of pharmacognostical studies made on roots of botanically authenticated plants of *Veratrum viride* Aiton, *V. album* L., *V. Woodii* Robbins ex Gray, *V. Eschscholtzii* A. Gray, *V. californicum* Durand, *V. fimbriatum* A. Gray, and on an undetermined species of *Veratrum* from Montana.

2. The rhizomes and roots of all of the aforementioned species of *Veratrum* excepting *V. fimbriatum* have been detected in drug commerce as substitutes for *Veratrum viride*.

3. Descriptions of the microscopic structure of each of the aforementioned *Veratrum* roots are given.

4. In the roots of all of the *Veratrums*, the outer walls of the epidermal cells were found to be convex and thickened, and the epidermal cells of *Veratrum viride*, *V. album*, *V. californicum*, and *V. fimbriatum* showed lignification at varying levels above the root cap in the mature regions.

5. The air spaces in the aerenchyma region of the cortex were radially elongated in all of the species studied excepting *V. Woodii*, where they were tangentially elongated. The aerenchyma occupied up to one-third the width of the cortex in *V. album*, *V. Eschscholtzii*, *V. Woodii*, and in the *Veratrum* from Montana, up to one-half in *V. viride*, two-thirds in *V. californicum*, and was most pronounced in *V. fimbriatum*, extending to the stele.

6. The starch grains were generally single, spheroidal, and 2- to 3-compound, the individual grains being mostly up to 11.5 μ in diameter in *Veratrum Woodii*, up to 19 μ in *V. album*, up to 20 μ in *V. viride*, up to 16.5 μ in *V. Eschscholtzii*, usually up to 16.5 μ, rarely up to 25 μ in *V. californicum* and in a *Veratrum* from Montana, and up to 26.4 μ in *V. fimbriatum*.

7. Raphides were observed in all of these roots, with the exception of *V. fimbriatum*, and their respective lengths are reported.

8. In cross sections, the endodermis consisted of one row of cells, quadratic to slightly radially elongated, the radial and inner walls of which were usually thicker than the outer walls. The lumina of these cells were mostly U-shaped in *V. viride* (though in some roots occasionally

[1] Since the presentation of this paper, flowering and fruiting plants of the unknown Montana *Veratrum* from around Missoula, Mont., were compared by the author at the Gray Herbarium with herbarium sheets of western *Veratrums* including *V. viride*, *V. Eschscholtzii*, etc., and with *V. viride* from Quebec and various stations in eastern United States. It was found that the Montana plants agreed in flower structure with the eastern North American *V. viride*, matching the Quebec plants rather closely in robustness of growth as well as in floral parts and in the size of the capsular fruits. Since the differences between eastern and western *V. viride* plants are to be found in the root and possibly other vegetative organs, it appears that the unknown *Veratrum* from Missoula, Mont., represents a western variety or form of *Veratrum viride*.

V-shaped or irregularly quadrangular), U-shaped in *V. Eschscholtzii*, *V. fimbriatum*, and in a *Veratrum* from Montana, both U- and V-shaped in *V. album*, *V. californicum*, and *V. Woodii*. The radial and inner walls of the endodermal cells showed lignification excepting in *V. fimbriatum*.

9. The radial, polyarch fibrovascular bundle in each of these roots varied in the number of phloem and of xylem strands. The range of each of these was from 8 to 14 in *V. viride*, 8 to 15 in *V. album*, 10 to 15 in *V. Woodii*, 10 to 20 in *V. californicum*, 9 to 15 in *V. Eschscholtzii*, 12 to 16 in a *Veratrum* from Montana, and 13 to 17 in *V. fimbriatum*.

10. Thirty-two (2n) somatic chromosomes were observed in fixed and stained root-tip materials of both *Veratrum viride* and *V. album*.

11. The *Veratrum* from Missoula, Mont., while differing somewhat in its root structure from eastern *V. viride*, agreed with the latter in its stable floral parts. It therefore appears to be a western form or variety of *Veratrum viride*.

12. The paper is supplemented by drawings.

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In Vitro Differences Between Dihydroxy Aluminum Aminoacetate and Dried Aluminum Hydroxide Gel*

By ROBERT S. MURPHEY

The prompt and prolonged acid-buffering action of dihydroxy aluminum aminoacetate is contrasted with the relatively slow action of dried aluminum hydroxide gel by two methods *in vitro*. It is also shown that dried aluminum hydroxide gel becomes less reactive toward dilute acid with increasing age, and that it is inhibited in its antacid action by pepsin. Dihydroxy aluminum aminoacetate neither loses activity with increasing age nor is its antacid action significantly inhibited by pepsin.

IT CAN BE CONSIDERED as established that dried aluminum hydroxide gel U. S. P. from different sources varies significantly in chemical and physical properties (1–3). Having observed "the lag in acid-adsorbing and neutralizing capacities of various samples of dried aluminum hydroxide gel," Krantz and his associates (4) first prepared dihydroxy aluminum aminoacetate (DAA) and showed it to have "immediate buffer capacity" and "prolonged acid-consuming capacity."

The U. S. P. method for determining acid-consuming capacity does not disclose either differences in the properties of U. S. P. dried gels or the rapid and prolonged acid-buffering capacity of DAA. The promptness of buffering and the effective *p*H range of the buffering agent as determined *in vitro* are of great importance, for it has been shown by Flexner and Kniazuk (5) and by Rossett and Flexner (6) that *p*H changes as determined by certain methods *in vitro* are an almost exact duplication of the *p*H changes observed in experimental animals and man when a given dose of antacid is administered. The *in vitro* method is capable of demonstrating both the extent of *p*H change and the time required for acid neutralization in the gastrointestinal tract of man when the antacid is administered as a therapeutic agent. Obviously, the therapeutic value of the antacid is determined by its ability to neutralize acid rapidly and for long periods of time.

In proposing a new U. S. P. test for antacids which takes into consideration the extent of *p*H change and the time required for buffering of acid, Holbert, Noble, and Grote (1) showed that DAA tablets are considerably more rapid in their buffering of acid than two brands of

* Received November 29, 1951, from the Research Laboratory, A. H. Robins Co., Inc., Richmond, Va.

aluminum hydroxide tablets. In a later paper (2) in which they reported on the prolonged buffering action of 10 antacid materials, they found that DAA was both rapid and prolonged in its buffering action, while only one of two brands of aluminum hydroxide exhibited a prolonged effect of comparable magnitude. In their first paper (1) these authors commented on the need for a standard artificial gastric juice for evaluation of gastric antacids *in vitro*. Obviously, the *p*H of such an artificial fluid is important, but equally important is the concentration of pepsin, since dried aluminum hydroxide gel is markedly inhibited in its antacid action by this enzyme.

Using a somewhat different method, Hammarlund and Rising (3) studied the buffering properties of 24 commercially available antacid materials. They also found that DAA is both rapid in action and prolonged in effect, while aluminum hydroxide gel powders are "rather slow acting but their effect is prolonged." Applying a modification to the method used by these authors (3), evidence has been obtained which indicates a loss in reactivity by aluminum hydroxide gel powder with increasing age.

Although dried aluminum hydroxide gel is markedly inhibited in its antacid action by pepsin and appears to suffer loss of reactivity with increasing age, DAA suffers neither of these defects. While two groups of workers (1–3) report pronounced variation in reactivity of aluminum hydroxide from various sources, neither group examined the possibility of variation in activity of DAA. This point has been examined by the present author by two methods (2, 3) and it is found that DAA as incorporated in one commercial form of this antacid[1] is uniform in reactivity from lot to lot.

EXPERIMENTAL

All experiments were carried out using a 250-cc. beaker equipped with a mechanical stirrer. Electrodes from a Beckman Model G *p*H meter were continuously immersed in the medium. In all cases, antacid powder ground to pass a 100-mesh screen was used, and weights of the antacid taken were dry weights based on the aluminum analysis of the material. Use of the antacid as a powder greatly changes the ratio of effectiveness of these two antacids as compared to the unbroken tablets, since many aluminum hydroxide tablets are exceedingly slow to disintegrate.

Method I.—This is a modification of the method employed by Hammarlund and Rising (3). One gram of antacid was added to 25 cc. of 0.100 *N* hydrochloric acid at 25°, and the *p*H was recorded at thirty seconds, at two minutes, and each two minutes thereafter until thirty minutes had elapsed. Twenty-five cc. of 0.100 *N* acid was added at the end of this time and the *p*H was followed as before. This process was repeated over a three-hour period. Figure 1, a graphical representation of the results obtained with this method, is discussed below.

Method II.—This is a modification of the method used by Holbert, Noble, and Grote (2). Two grams of antacid powder was added to an artificial gastric juice (150 cc. of *p*H 1.50 hydrochloric acid containing either 0, 1.0, or 2.0 Gm./L. of pepsin, N. F.). After each ten-minute period, 20 cc. of the mixture was removed and 20 cc. of fresh juice was added. The *p*H was recorded at thirty seconds and at two, four, six, eight, and ten minutes in each ten-minute period over a two-hour span. The results obtained by this method with 0, 1, or 2 Gm./L. of pepsin are shown in Figs. 2, 3, and 4, respectively.

DISCUSSION

On a dry basis, DAA contains 20.0% aluminum while aluminum hydroxide contains 34.6% aluminum. Therefore, aluminum hydroxide contains 73% more aluminum than does DAA. All comparisons, however, have been made between equal weights of the two antacids.

Figure 1 shows the marked buffering action of DAA as compared with samples of dried aluminum hydroxide gel of two and eighteen months of age. Eighteen-month-old DAA also is plotted for comparison. Attention is called to the fact that DAA continues to buffer added acid promptly, even after sufficient acid has been added to completely react with the free amino group, for which 74 cc. of 0.100 *N* acid per gram is required. While the results of Hammarlund and Rising (3) reveal the initial prompt buffering action of DAA as compared with dried gel, they do not show the continued prompt buffering action; and one might wonder if the initial action was not due to the fact that DAA was more readily wet by the solution than was the dried gel. The present results (see all figures) show that the prompt action is inherent in the molecule and is not due to particle size or difference in wetting properties.

In Fig. 1, a prominent dot on each thirty-minute line points out the *p*H which should result from each addition of acid if the true buffering capacity of the antacid is exhausted. It is seen that the dried gel is very slow in its reaction to each addition of acid in comparison with the rapid reaction of DAA. It is generally agreed that maintenance of a *p*H range of 3.5 to 4.5 is desirable in antacid therapy, and if one totals the time these antacids hold the *p*H above 3.5 over the three-hour test period by Method I, the results which are tabulated in Table I are obtained.

That loss of reactivity of the dried gel with age might occur was suggested by a study of the diffraction patterns of α- and β-aluminum hydroxide gels by Geiling and Glocker (8). They stated that the α-gel ages on standing with a gradual transition, incomplete in seven weeks, to the β-gel, which shows some of the interferences characteristic of boehmite. Bassett and Durant (9) have stated that only the amorphous aluminum hydroxide dissolves readily in dilute acid. The results obtained may be explained

[1] Robalate tablets (dihydroxy aluminum aminoacetate, N. N. R.), A. H. Robins Co., Inc., Richmond, Va.

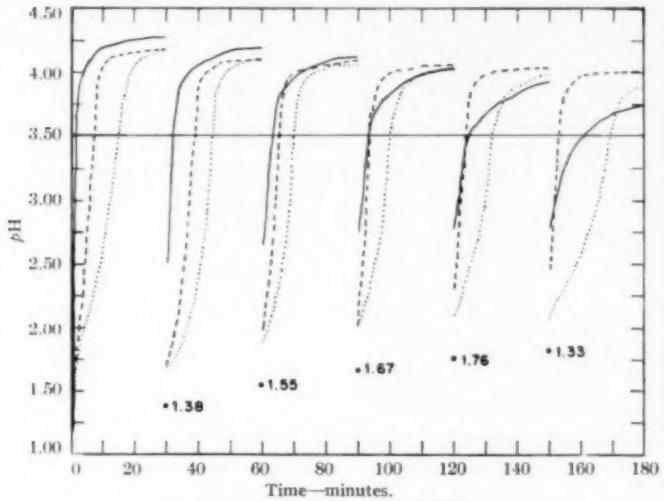

Fig. 1.—Buffer action by Method I.

——— DAA, 18 months. – – – – Dried Aluminum Hydroxide Gel, 3.5 months. . . . . Dried Aluminum Hydroxide Gel, 18 months.

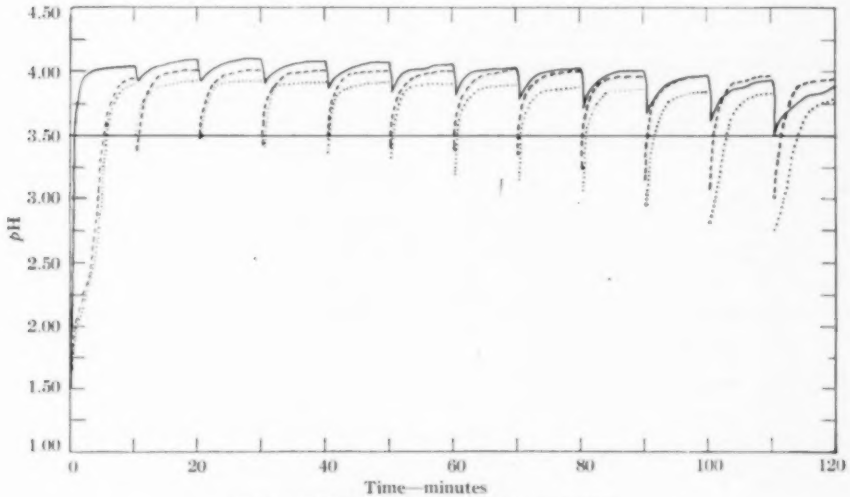

Fig. 2.—Buffer action by Method II, no pepsin.

——— DAA, 18 months. – – – – Dried Aluminum Hydroxide Gel, 2 months. . . . . Dried Aluminum Hydroxide Gel, 18 months.

on the basis of continuing crystallization of the dried gel. The results obtained with antacid 7 in Table I emphasizes the point that the dried gel need not be old to exhibit poor reactivity to acid. All of the dried gels listed in Table I passed the U. S. P. test for acid-consuming capacity.

The poor antacid action of aluminum hydroxide gel in humans was reported by Rossett and Flexner (6) and by Kirsner and Palmer (7). A partial explanation for the poor behavior of aluminum hydroxide gel in the body as contrasted with its good antacid action when examined by methods employing only acid *in vitro* may be found in the pronounced inhibition of its antacid action by pepsin. Other proteins, such as egg albumin and hemoglobin, have only a slight retarding effect on aluminum hydroxide.

The Rossett and Flexner *in vitro* method (6) was made more stringent by Holbert, Noble, and Grote

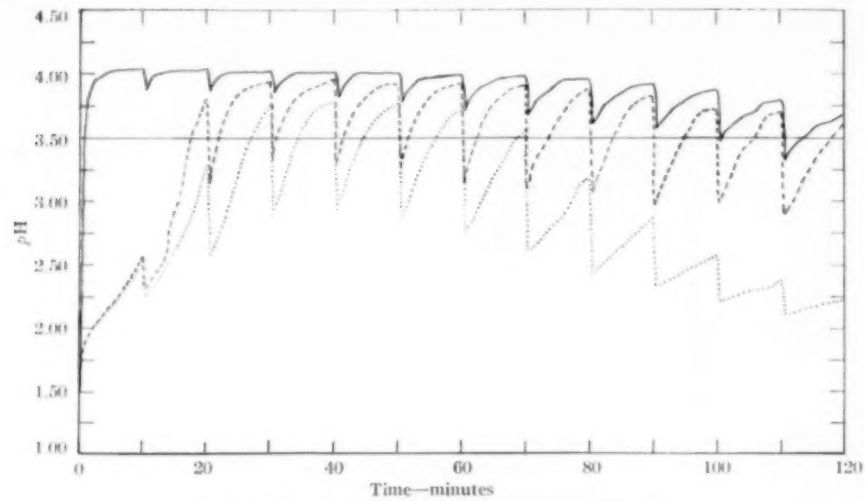

Fig. 3.—Buffer action by Method II, 1.0 Gm./L. of pepsin.
——— DAA, 18 months. – – – – Dried Aluminum Hydroxide Gel, 2 months. . . . . Dried Aluminum Hydroxide Gel, 18 months.

(1), who incorporated the feature of removal of part of the acid-antacid mixture at definite intervals to simulate the loss of material from the stomach to the duodenum. The results obtained by the present author using this method (1) with 2.0 Gm./L. of pepsin N. F. (see Fig. 4), present a better picture for fresh dried aluminum hydroxide gel and a similar picture for 18-month-old dried aluminum hydroxide gel when compared with the results reported for the *in vivo* effect of this antacid (6, 7). Hackett (10) has correlated commercial pepsin with gastric pepsin, and calculations lead to a figure of 1.8 Gm./L. of pepsin in gastric juice, although several artificial gastric juice formulas use 3–5 Gm./L.

TABLE I.—TOTAL TIME *p*H HELD ABOVE 3.5

No.	Antacid	Age, Mo.	Total Time, Min.
1	Dihydroxy aluminum aminoacetate[a]	2	156
2	Dihydroxy aluminum aminoacetate	18	148
3	Dried aluminum hydroxide gel[a]	2	154
4	Dried aluminum hydroxide gel	3.5	145
5	Dried aluminum hydroxide gel[a]	12	109
6	Dried aluminum hydroxide gel	18	92
7	Dried aluminum hydroxide gel[a]	3	34

[a] Not shown in Fig. 1.

Figure 2, representing the behavior when no pepsin is present, reveals only one significant difference between the two antacids under consideration. The initial and continued rapid buffering action of DAA stabilizes the *p*H more than does that of the more slowly acting dried gels. A summary of the effect of pepsin concentration on the two antacids is given in Table II.

The precipitation of pepsin by colloidal aluminum hydroxide was reported by Komarov and Komarov (11) who mentioned that some of the antacid was probably carried down in the characteristic protein-like precipitate. They stated that the precipitate dissolved in dilute acid of the concentration normally occurring in the stomach (*p*H less than 2), thus releasing active pepsin at the site of the precipitate. Shay, *et al.* (12), called attention to the undesirability of this reversible precipitation of pepsin since reactivation of the pepsin by acid in an ulcer crater might cause the lesion to progress toward perforation. Schiffrin and Komarov (13) state that the

TABLE II.—EFFECT OF PEPSIN CONCENTRATION

	DAA			Dried Aluminum Hydroxide Gel					
Age (Months)	18	18	3[b]	2	2	2	18	18	18
Pepsin, Gm./L.	0	1	2	0	1	2	0	1	2
Min. to pass *p*H 3.5	½	1	2[b]	6	18	48	6	28	180+[a]
Final *p*H	3.88	3.68	3.50[b]	3.94	3.61	2.74	3.78	2.23	2.04

[a] Maximum *p*H was 2.62.
[b] As Robalate.

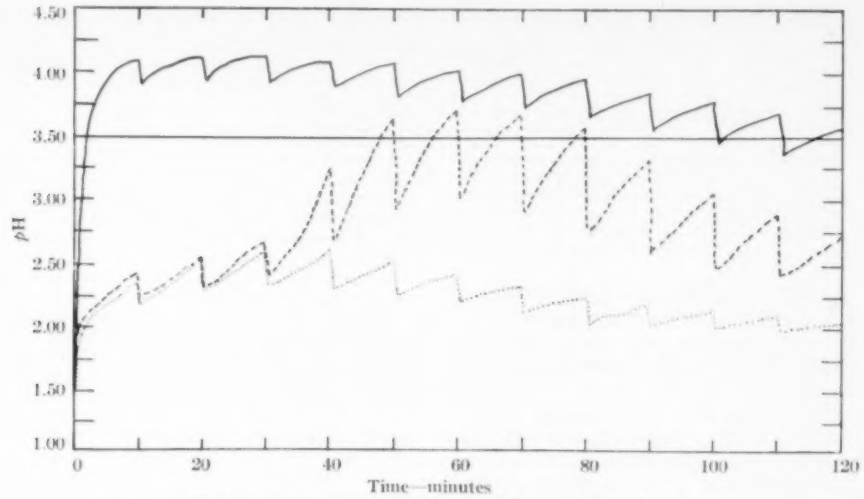

Fig. 4.—Buffer action by Method II, 2.0 Gm./L. of pepsin.
——— Robalate, 2 months. – – – – Dried Aluminum Hydroxide Gel, 2 months. . . . . Dried Aluminum Hydroxide Gel, 18 months.

aluminum ion inhibits pepsin without precipitation. While DAA does not precipitate pepsin, it is capable of inactivating it by prompt and prolonged elevation of the *p*H and by the prompt contribution of aluminum ions to the gastric solution.

SUMMARY

1. Dried aluminum hydroxide gel appears to decrease in reactivity to acid with increasing age, while dihydroxy aluminum aminoacetate is not significantly affected by time.

2. Dried aluminum hydroxide gel is greatly inhibited in its antacid action by pepsin while the buffering property of dihydroxy aluminum aminoacetate is not appreciably diminished by this enzyme.

3. The previously reported rapid and prolonged buffering action of dihydroxy aluminum aminoacetate in the optimal *p*H range of 3.5 to 4.5 is confirmed by two *in vitro* methods.

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Solubilizing Effects of Calcium Salts upon Calcium Gluconate*

By BERNICE MARCH, L. O. SOMMERS, and E. E. MOORE†

Evidence is presented which indicates that the formation of new compounds is accountable for the increase in solubility of calcium gluconate in the presence of other more soluble calcium salts. The preparation of these compounds is given.

ALTHOUGH the solubility of calcium gluconate is only about 3 per cent, solutions containing 10 per cent often remain unchanged for long periods of time if prepared absolutely free of undissolved particles and other foci of crystallization. Since this is not always feasible, attempts to prepare stable concentrated solutions by the addition of various stabilizing agents have occupied a great deal of study. Practically every soluble and one slightly soluble calcium salt has been included in the list of compounds reported to stabilize calcium gluconate solutions. The concentration of these salts has usually been as little as 1 per cent or even less. However, investigators have reported that in such cases as calcium lactobionate (1) and calcium methionate (2) equimolar quantities must be used.

In the present work a quantitative study of the increased solubility of calcium gluconate in the presence of the one slightly soluble and several soluble calcium salts have been made, and from the evidence obtained an explanation of this increase in solubility by the formation of other stable soluble compounds is presented. The observed increase in solubility never exceeded that attributable to the amount of the combined product formed by the reaction of calcium gluconate with an equimolar amount of the other more soluble calcium salt.

EXPERIMENTAL

The new calcium compounds were prepared by the following methods:

1. An aqueous mixture of equimolar quantities of the calcium salts was allowed to reflux for one hour.

2. An aqueous mixture of equimolar quantities of one acid and the calcium salt of the other acid was neutralized by the addition of calcium carbonate and stirred at 80° for one hour.

3. An aqueous solution of equimolar quantities of the two acids was neutralized with calcium carbonate and stirred at 80° for thirty minutes.

The solid compounds were then isolated by precipitation with methanol or by evaporation of the solution to dryness under vacuum. It is to be noted that the reaction is much slower with the component salts than with the corresponding acids and calcium carbonate.

As the above procedures are essentially similar, only one example need be given in detail.

Calcium Gluconate-Calcium Glutamate Dihydrate.—Calcium gluconate 21.5 Gm., glutamic acid 14.6 Gm., and calcium carbonate 8 Gm. in 380 cc. of water were heated with stirring at 80° for one hour. The undissolved calcium carbonate was removed and the filtrate concentrated to 200 cc. under reduced pressure and poured into 300 cc. of methanol, from which the resulting compound separated. It was collected and dried under vacuum at 85°. The compound was then redissolved in 100 cc. of water, filtered, and evaporated to dryness under vacuum at 85°. The yield was 26 Gm.

Anal.—Calcd. for $C_{11}H_{19}O_{11}NCa \cdot 2H_2O$: C, 31.65; H, 5.55; Ca, 9.60. Found: C, 31.62; H, 5.58; Ca, 9.58.

Soluble compounds of calcium gluconate with calcium glutamate, calcium levulinate, and calcium glucoheptonate have been prepared by the above methods. None was found to combine with calcium gluconate in greater than a mole-to-mole ratio. Calcium *d*-saccharate, which is very slightly soluble, did not form a more soluble salt with calcium gluconate.

Solubility Determinations.—Solutions (10%) of each of the above-mentioned, isolated compounds were tested for supersaturation with respect to calcium gluconate by the following method. A bacterin-type vial was filled with the solution to be tested, stoppered, and sterilized. Sterile crystals of calcium gluconate were transferred aseptically to the solution from an ampul of 10% calcium gluconate in which crystallization had occurred. A solution (10%) of calcium gluconate formed a heavy precipitate; all of the solutions containing calcium gluconate in combination with the soluble calcium salts remained clear.

Equimolar mixtures of the two calcium salts making up each of the combined compounds were then prepared (*a*) by intimately mixing equimolar quantities of the dry component salts, and (*b*) by precipitating with methanol an aqueous equimolar solution of the two salts that had been boiled for only ten minutes. Each of the resultant solids showed less than 10% water solubility, as compared to a solubility of over 30% by weight for most of the combined compounds prepared by the methods described previously.

In order to determine the minimum amount of soluble calcium salt required to solubilize calcium gluconate, a series of mixtures containing various molar ratios of the two salts in question were prepared. Aqueous solutions of these mixtures were

* Received December 3, 1951, from Abbott Laboratories, North Chicago, Ill.

† The authors wish to thank the Microanalytical Department for the analyses.

refluxed for several hours and then evaporated to dryness. The approximate solubilities of the oven-dried residues were determined in the following manner. A weighed sample of the residue was placed in a cylinder which was equipped with a burette, a mechanical stirrer, and a condenser. The system was held at 25° while water was added very slowly from the burette, with stirring, until the solid had dissolved completely. The volume of water added and the density of the solution were determined; from these values and the weight of the sample the solubility was calculated. The results so obtained are reproducible. The solubility data for calcium gluconate-calcium glucoheptonate as an example are reported on an anhydrous basis in Table I.

The fact that calcium glucoheptonate enhances the solubility of calcium gluconate and probably combines with an equimolar amount is shown in Table I. In column C the molar concentration of calcium gluconate (after reaction with calcium glucoheptonate) approaches the solubility of unreacted calcium gluconate as the amount of calcium glucoheptonate is decreased. In column E it is seen that the molar concentration of calcium gluconate less that of calcium glucoheptonate does not exceed the solubility of the uncombined calcium gluconate. The solubility of the latter is actually depressed as the total solids in solution are increased.[1] This suggests strongly that calcium gluconate and calcium glucoheptonate combine in a mole-to-mole ratio.

TABLE I.—SOLUBILITIES OF CaG_2[a]-$CaGh_2$[b] COMBINATIONS

A Mole Ratio CaG_2:$CaGh_2$	B Total Solubility, Gm./L.	C Molar Concn., $CaG_2 \cdot 10^3$[c]	D Molar Concn., $CaGh_2 \cdot 10^3$[d]	E (C − D)[e] Molar Concn. Uncombined $CaG_2 \cdot 10^3$[d]
1.25:1[f]	83.99	10.21	8.18	2.03
2:1	55.60	8.24	4.12	4.12
4:1	40.54	7.34	1.83	5.51
8:1	33.92	6.90	0.86	6.04
1:0	27.28	6.34	...	6.34

[a] Calcium gluconate.
[b] Calcium glucoheptonate.
[c] Present in both combined and uncombined forms.
[d] Present as combined in the soluble compound.
[e] Assuming a 1:1 mole ratio in the soluble compound.
[f] Calcium glucoheptonate alone forms a highly concentrated syrup with water which makes the solubility determination very difficult. The compound resulting from a 1:1 ratio was found in a rough determination to be soluble in excess of 300 Gm./L.

DISCUSSION

The increased solubility of the refluxed products shows that they are unlike their component salts when prepared as a mechanical mixture or precipitated from a solution after short boiling. Further data confirm the conclusion that definite compound formation occurs. For example, to form the soluble product from calcium gluconate and another more soluble calcium salt, prolonged heating, which is not necessary for a simple mixture, is needed. The solubility data indicate a stoichiometric relationship between the two reactants corresponding to a mole-to-mole reaction. Increased solubility of calcium gluconate was found to occur only when it reacted with another calcium salt of greater solubility. The resulting compound did not have as great a molar solubility as the more soluble calcium salt.

Although the evidence points to the formation of new compounds, their nature and structure are not yet clear. Physical data, now being gathered, will fully characterize the crystals which have been isolated, and will be presented in a second paper. In addition, the results of tests now in progress aimed towards an elucidation of the structure of these new entities will be included. It is believed that these data will explain clearly the reasons for the increased solubility of calcium gluconate reported in the present paper.

[1] It is expected that the solubility of uncombined calcium gluconate would be depressed due to a salting-out and/or common ion effect of the combined product.

SUMMARY

Calcium gluconate combines with many soluble organic salts to form readily soluble products. A number of these have been prepared and examined. The maximum increase in solubility of calcium gluconate was attained when at least an equimolar amount of the more soluble salt was present. In this study the molar solubility of the resulting compound was never found to exceed that of the more soluble calcium salt.

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The Determination of the Stability of Vitamin B_{12} in Multivitamin Mixture by a Radioactive Indicator Method*

By CHARLES ROSENBLUM and D. T. WOODBURY

By means of cobalt 60 labeled vitamin as radioactive tracer, the stability of vitamin B_{12}, after thirteen months' storage in four different multivitamin combinations, has been determined. The cobalt 60 labeled vitamin B_{12}, prepared by microbial synthesis, was extracted from the multivitamin preparations by using three separate solvents, and determined by its radioactivity.

THE DETERMINATION of stability of a compound is essentially an analytical problem involving periodic analysis for the substance in question either in the pure state or in a matrix in which it is incorporated. Required for such an evaluation are (*a*) a reliable analytical method, and (*b*) a zero-time base from which to judge the extent of subsequent change, if any.

Both requirements become more difficult of realization when the subject compound is a complicated, biologically active substance such as a vitamin or antibiotic incorporated in a complex mixture of salts and naturally occurring materials, as may be encountered in certain types of pharmaceutical preparations. A case in point is the stability of vitamin B_{12} (cyanocobalamin) in multivitamin capsule material derived from liver concentrates which may normally contain appreciable quantities of vitamin B_{12}. The microbiological assay of such a medium for B_{12} content is of limited accuracy, and, although the B_{12} analysis based on the determination of cyanide (1) has been applied successfully to liver concentrates, the entire problem can be simplified by radioisotope tracer methodology.

Both conditions for a stability determination can be satisfied unequivocally by means of an isotopically labeled compound, as has been done with vitamin B_{12} labeled with cobalt 60 (2, 3). A given amount (weight and radioactivity) of the radiovitamin was mixed with capsule materials at a concentration level of 10 γ per capsule, and these preparations stored at room temperature for varying periods of time. After a given storage period, the vitamin was extracted and the total radioactivity measured. It was not necessary to obtain the vitamin in a pure state, but only to separate it from possible decomposition products of the added radiovitamin, nonradioactive endogenous impurities being of no consequence in this method. The radioactivity of the extract was shown to be integral with vitamin B_{12} by measuring its distribution coefficient. Zero storage-time blanks were also run to determine the distribution coefficient to be expected, as well as the extraction efficiencies attainable. Four different proprietary capsules were investigated. The maximum storage time studied was about thirteen months. Considerable B_{12} stability was noted in all cases, despite the complexity of the matrix.

* Received February 2, 1952, from the Research and Development Division, Merck & Co., Inc., Rahway, N. J.

EXPERIMENTAL

Materials.—The labeled compositions of the four multivitamin capsule preparations studied are listed in Table I. One of the preparations (A) contained ferric iron, two (B and D) were enriched with a ferrous salt, while no iron was added in the formulation of capsule C.

The cobalt 60 labeled radiovitamin employed was prepared by microbial synthesis (2). It had a specific activity of about 0.25 microcurie per milligram. Countercurrent analysis by radioactivity and color indicated a minimum purity of 95%.

Sample Preparation.—Each sample in a series was prepared by expressing the contents of five capsules into a 40-ml. centrifuge tube. To each tube 0.25 ml. of a methanolic solution of radioactive vitamin B_{12} was added, and the contents stirred to distribute the tracer uniformly. The amount of vitamin added per sample was 50 γ, having a radioactivity of about 1,800 c.p.m., as measured in the equipment employed. The sample tubes thus prepared were pumped at room temperature for about thirty minutes to remove the methanol. The tubes were then stored at room temperature in brown glass bottles until required for B_{12} extraction. A separate tube was prepared for each time studied, including the zero-time experiments.

Extraction Procedure.—The vitamin B_{12} left at any given time was determined by extracting first with methanol. The extraction was performed by adding five successive 10-ml. portions of methanol sequentially to a sample centrifuge tube. After each addition of methanol the sample was stirred for five minutes, while the centrifuge tube was immersed in a hot water bath (65–70°). The tube was then centrifuged slowly, and the methanolic supernatant liquid decanted into a filter cone. The five successive filtrates so obtained were collected in a 50-ml. volumetric flask and diluted to volume with methanol. Of this solution, 4 ml. was removed for radioactivity

TABLE I.—COMPOSITION OF CAPSULE CONTENTS

Component	Quantity per Capsule	Component	Quantity per Capsule
Capsule A		**Capsule B**	
Liver-stomach concentrate	0.455 Gm.	Liver-stomach concentrate	0.455 Gm.
Iron and ammonium citrates, green	0.2 Gm.	Ferrous sulfate, anhydrous	0.1 Gm.
Thiamin chloride	0.15 mg.	Thiamin chloride	0.15 mg.
Riboflavin with other factors of vitamin B complex present in liver-stomach concentrate	0.05 mg.	Riboflavin with other factors of vitamin B complex present in liver-stomach concentrate	0.05 mg.
Capsule C		**Capsule D**	
Thiamin HCl	2 mg.	Thiamin HCl	0.3 mg.
Riboflavin	2 mg.	Ferrous sulfate, dried, U. S. P.	136.1 mg.
Pyridoxine HCl	0.125 mg.	Whole dried liver	194.4 mg.
Niacinamide	10 mg.	Vitamin B_{12}	5 μg.
Calcium pantothenate	2.18 mg.		
Liver 1:20	162 mg.		
Yeast, dried, U. S. P.	162 mg.		
Vitamin B_{12}	1 μg.		

assay to determine the percentage of activity extracted at this stage. The remaining 46 ml. was transferred to a 500-ml. round-bottom flask washed with two 3-ml. portions of methanol to effect complete transfer.

Carrier vitamin B_{12} (1 mg.) was added to the methanolic solution at this point, and the solution evaporated to dryness by pumping with the flask immersed in a hot water bath (approx. 65–70°). The residue was dissolved in a few milliliters of water and transferred to a 10-ml. volumetric flask, using several rinses of 1–2 ml. each to effect the transfer. Of the 10 ml. of aqueous extract, 1 ml. was used for radioactivity measurement to determine the radioactivity recovered at this step. The remaining 9 ml. was transferred to a 30-ml. separatory funnel and an equal volume of saturated ammonium sulfate solution added. The 18-ml. extract, half-saturated with ammonium sulfate, was then extracted by shaking (one minute) twice with 2-ml. portions of benzyl alcohol, and once with a 1-ml. portion of the alcohol. In each case the benzyl alcohol phase separated rapidly from the aqueous phase. The benzyl alcohol extracts were combined in a 15-ml. centrifuge tube and centrifuged to separate suspended droplets of the aqueous salt solution. The volume of the benzyl alcohol phase was usually slightly in excess of 5 ml., probably due to the solubility of water (and capsule components) in the benzyl alcohol. In most cases, 1 ml. of the benzyl phase was removed for radiometric assay. This permitted a check of the activity recovered at the third stage of the extraction process.

Distribution Test.—A 4-ml. portion of the benzyl alcohol extract was delivered to a 15-ml. centrifuge tube and 4 ml. of water added. The tube was stoppered with a polyethylene stopper, shaken for one minute and centrifuged. Aliquots of 2 ml. of each phase were employed for radioactivity analysis. The distribution coefficient of extracted radioactive component was calculated from the ratio of c.p.m.'s in the two phases. These data could also be used to check calculation of percentage of activity recovered at this point.

Additional capsules of each type were extracted through the benzyl alcohol stage, without prior addition of tracer, to permit a measurement of the distribution coefficient of vitamin B_{12} uncomplicated by storage contact with capsule components. The zero-time tubes prepared as blanks actually were about one hour old before extraction was begun, and the extraction itself required about two hours to reach the benzyl alcohol stage. An unequivocal determination of the distribution coefficient was obtained by shaking an aqueous solution of radiovitamin with the above benzyl alcohol extracts, and determining the radioactivity of each phase.

Radioactivity Measurements.—Aliquots of the various solutions were evaporated in 1-in. stainless-steel cupped planchets under an infrared lamp, and the radioactivity of the residues measured with a thin mica window Geiger-Mueller tube. The diameter and thickness of the window were 3.0 cm. and 2.1 mg./cm.^{2}, respectively. The samples were maintained at a fixed distance of 13 mm. from the window during measurement. A self-absorption curve (4) pertaining to the system was constructed, and appropriate corrections made as required. Planchets containing cobalt 60 were prepared to serve as a standard for detecting and correcting for daily variations in G-M tube sensitivity. The over-all efficiency of the counting equipment was about 6%. All counts were made with a standard deviation of about 2%.

RESULTS

Results are tabulated in Table II. In column 3 is given the average value of the successive extraction efficiencies with methanol and water. Column 4 lists the extraction efficiencies at the benzyl stage. Column 5, "Net" extraction efficiency, is the product of the two preceding figures, and represents the per cent of added B_{12} (cobalt 60 labeled) which is recoverable as such after the indicated storage period. It is noted from the zero-time (or four-day) experiments that the extraction method is quite effective (94–95%) in removing the vitamin from capsules A and B, but only 78% efficient in the case of capsules C and D. The low extraction efficiency occurred primarily at the initial methanol treatment, subsequent extraction with water and transfer to benzyl alcohol being essentially quantitative with unaged preparations. Accordingly, a correction has been made for the inefficiency of the initial extraction by normalizing "Net" recoveries to the above cited

TABLE II.—STABILITY OF VITAMIN B_{12} IN CAPSULE MATERIAL

Capsule	Storage Time, Days	% Radioactivity Extracted: Methanol; Water[a]	Benzyl Alcohol	Net	% B_{12} Present (Normalized)	Distribution Coefficient
A	0	94	..	..	..	1.23
	4	96	99	95 | 94.5	100	1.19
	4	97	97	94 /		1.35
	49	96	84	81	86	1.45
	96	99	84	83	88	1.28
	228	91	86	78	83	1.25
	412	85	89	76	80	1.29
B	0	95	99	94	100	1.33
	42	99	80	79	84	1.32
	96	103	83	85	90	1.39
	228	98	82	80	85	1.22
	412	86	89	77	82	1.31
C	0	77	101	78	100	1.18
	7	78	104	81	104	1.13
	104	78	95	74	95	1.25
	231	72	97	70	90	1.14
	413	59	91	54	69	1.44
D	0	86	91	78	100	1.68
	104	87	90	78	100	1.33 (?)
	231	90	89	80	102	1.76
	413	72	99	71	91	1.67

[a] Average of methanol and water values (except first result in table, which is methanol only).

initial recovery efficiencies as representing 100%. Such corrected values of "% B_{12} present (normalized)" are listed in column 6. Measured distribution coefficients (water/benzyl alcohol) are shown in the last column.

Detailed information for an illustrative run is compiled in Table III to demonstrate the method of treating data. The efficiency of extraction of radioactivity at the methanol and water stages (columns 2 and 3) were found to be 85.5 and 85.3%, respectively, by comparing the total c. p. m. recovered (correcting the water figure for the 4 ml. of methanol extract taken for assay) with the added c. p. m. Good agreement between these two measurements was obtained in almost every case reported in Table I, since the water extraction is essentially quantitative and simply reflects the success of the methanol treatment. The transfer of radioactivity to benzyl alcohol upon addition of salt to the aqueous extract amounted to 89.0%. This was calculated by comparison of the total c. p. m. recovered with the activity of the water phase (9 ml.) extracted. In many cases, however, the benzyl extract was not sampled directly. An alternate calculation of the efficiency of the alcohol extraction could be made from the distribution experiments since the sum of the "total c. p. m. recovered" valuesf or the benzyl and water phases is a measure of the radioactivity extracted by the organic solvent. The transfer of radioactivity calculated in this manner is 88.8%, which agrees with the results of direct measurement.

DISCUSSION

Judging solely from the radioactivity extraction measurements summarized in Table II, and admitting the validity of the normalized "% B_{12} Present" values calculated therefrom, one must conclude that about 80% of the B_{12} added initially to capsules A and B is recoverable as such, even after more than thirteen months of storage, and that half the loss occurs within the first one to two months. In the case of capsules C and D, the noted losses after four hundred and thirteen days of storage were but 31% and 9%, respectively, the marked change occurring only after seven to eight months of storage. One may say further, from A and B data, that the stability of vitamin B_{12} is the same in the presence of di- and trivalent iron salts.

Values of "% B_{12} present (Normalized)" in Table II were calculated from the column headed "Net % Radioactivity Extracted" by dividing values for a given time by the zero-time value in a given series of measurements. Implicit in such a computation is the assumption that low zero-time "Net" extraction values result from a physical inefficiency of the extraction method, rather than from a rapid chemical transformation of the radiovitamin in the capsule matrix. A corollary assumption requires the unextracted vitamin to have the same stability as the extracted material. There can be no objection to applying this correction to the data for capsules A and B, since zero-time extractions are essentially quantitative.

In the case of capsules C and D, which were only 78% extractable initially, the computation would seem to need some justification. Were this effect truly the result of vitamin instability rather than extraction inefficiency, one would have to assume a loss of about 20% of the vitamin in about one hour, and then stability for months, since the net extraction value remains relatively constant for many months. It is more reasonable to consider the extraction incomplete, and to base stability conclusions on the normalized % B_{12} values. It is possible that the nonextractable vitamin is bound in some manner with a component of capsules C and D. Vitamin B_{12} is known to be bound by certain proteins (5); and in the case of "intrinsic factor" (6, 7), the biological effectiveness of the bound vitamin is actually enhanced. It is thus possible that the stability values reported above are minimal, since any combination involving B_{12} to form a compound retaining its biological activity but which is not removable by the procedure employed would be overlooked.

TABLE III.—DATA FOR PREPARATION A, 412 DAYS OLD

	Methanol	Water	Benzyl Alcohol	Distribution: Water	Distribution: Benzyl Alcohol
Total volume, ml.	50	10	5.0	4	4
Volume in planchet, ml.	4	1	0.8	2	2
C. p. m. in planchet	121	139	178	250	194
Total c. p. m. recovered	1,513	1,510[a]	1,112	500	388
Theoretical initial, c. p. m.	1,770	1,770	1,251[b]		1,000[c]
% c. p. m. recovered	85.5	85.3	89.0		88.8
Distribution coefficient	...	...	...		1.29

[a] Corrected for aliquots previously removed.
[b] Assuming complete extraction of the radioactivity (1,390 c. p. m.) present in the 9-ml. aqueous extract.
[c] Calculated for 4 ml. of benzyl alcohol. This is the theoretical initial activity corresponding to that distributed between the water and benzyl alcohol phases.

Inspection of the measured distribution coefficients shown in the last column of Table II indicates that the radioactivity measurements truly represent vitamin B_{12}, since in each series the radioactivity extracted in the benzyl alcohol from the aged preparation distributes essentially the same as does the radioactivity extracted from the zero-time blanks. Thus, with few exceptions, the observed distribution remains constant within experimental error (±5%) within a series, and equal to that of the fresh (zero-time) samples, which presumably represent the behavior of unchanged vitamin B_{12} itself. Small variations reported probably represent, in part, differences in amount or nature of the extracted solute in the alcohol, or possibly *p*H differences.

The distribution coefficient (2, 8, 9) of vitamin B_{12} in the system water-benzyl alcohol is 1.2. This was confirmed with the radioactive vitamin preparaton employed in these experiments. The coefficients noted in the benzyl extracts differ somewhat from this figure, notably that of capsule D (about 1.7). That these deviations are due to an effect of extracted solute dissolved in the benzyl alcohol, i.e., a solvent effect, rather than to reaction of radiovitamin during storage contact with capsule components was demonstrated by the distribution coefficients obtained by shaking an aqueous solution of radiovitamin with the benzyl extract from normal (nonradioactive) capsule materials. Coefficients obtained were 1.2, 1.3, 1.24, and 1.63, respectively, for capsules A through D, which compare favorably with the averages 1.25, 1.31, 1.18, and 1.7 of experimental values reported in Table II.

The possibility that cobalt compounds closely related to the vitamin and derived therefrom may interfere with the B_{12} identification is not very likely. Free cobalt ion can be excluded because its distribution coefficient (water/benzyl) is in excess of 600. Furthermore, the most likely decomposition product, vitamin B_{12a} [aquo- or hydroxo-cobalamin] (10), has a high (8, 9) distribution coefficient (about 8); and any significant change of this nature would be revealed by higher observed distribution ratio. This may account for the 1.4+ figures obtained with forty-nine-day-old capsule A and four-hundred-and-thirteen-day-old capsule C. A distribution of 1.44 instead of 1.2 would correspond only to about 15% conversion of B_{12} to B_{12a}. These considerations do not apply to the 1.7 value obtained in three of the four runs with capsule D. This ratio is apparently due to B_{12} itself and not a result of conversion to a B_{12} analog.

SUMMARY

1. The stability of vitamin B_{12} in four different multivitamin capsule materials, after storage at room temperature for more than thirteen months, was determined by means of cobalt 60 labeled vitamin as radioactive tracer.

2. The tracer vitamin was extracted by a procedure involving three separate solvents.

3. The quantity of B_{12} extracted was determined by its radioactivity rather than by conventional chemical assays, and the identification of the radioactivity with the vitamin was made by determining its distribution in the system water–benzyl alcohol.

4. The maximum losses noted ranged between 9 per cent and 31 per cent in more than a year. The valence of the iron (ferrous or ferric) present in the capsule materials had no effect on the stability.

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Some Ultraviolet Absorption Characteristics of Amphetamine*

By ILSE EBSTEIN and C. T. VAN METER

The ultraviolet absorption characteristics of amphetamine have been investigated in various solvents and at different *p*H's. It has been found that the absorption is sufficiently intense in solutions containing 200 to 500 μg. of amphetamine per cc. to permit accurate determination. The important absorbing regions of the different spectra are depicted and specific absorbancy values are given for the various absorbancy peaks. The procedure can be adapted to a wide variety of amphetamine preparations.

AMPHETAMINE (α-methylphenethylamine), in the form of the free base and in the form of various salts of both the dextro- and racemic varieties, has been incorporated into a considerable number of simple and combination specialty products by various manufacturers. The quantity of the amphetamine component in these products is usually small in relation to the total quantity of other active and inactive ingredients. This often makes for difficulties in the assay of such preparations for their amphetamine content through adaptation of the pharmacopeial assay procedure which involves liberation of about 75 mg. of amphetamine, quantitative extraction of the free base with ether, cautious evaporation, over-neutralization with excess standard acid, and back-titration of the surplus acid with standard sodium hydroxide solution.

The problem came to the attention of this laboratory when we were confronted with the task of establishing adequate control specifications for a tablet product containing 2.5 mg. of dibasic *d*-amphetamine phosphate and 500 mg. of methylcellulose per tablet. It seemed reasonable to expect that amphetamine should absorb sufficient energy somewhere in the ultraviolet region to permit measurement by spectrophotometric assay, thus minimizing assay difficulties by making possible the employment of a smaller starting sample.

However, review of the chemical literature commonly employed in this country revealed that such data either have not been published or are not readily available. It was therefore decided to explore the ultraviolet region using selected solutions of the free base and of some of its salts. Data obtained are shown in Table I.

MATERIALS

Inasmuch as the main purpose of this investigation was to provide data which might be useful in the analysis of pharmaceutical preparations, the solutions selected for study were chosen with this primary aim in mind. For the free base, solutions in alcohol U. S. P., anhydrous chloroform, and anhydrous ether were investigated.

Salts of amphetamine commonly employed in the industry include the hydrochloride, the monobasic and dibasic sulfates, and the monobasic and dibasic phosphates. Partly because of its official status and partly because of its better physical characteristics as indicative of degree of purity, the dibasic *dl*-sulfate was selected for initial employment as the salt in this investigation. Solvents employed were water and 10 and 20% by volume alcohol. Aqueous solutions adjusted to *p*H 4, 3, and 2 were also studied. Adjustment to *p*H 4 and 3 was secured through the addition of HCl; the solution of *p*H 2 was prepared by using the appropriate HCl-KCl Clark and Lubs buffer mixture as the solvent.

EXPERIMENTAL

Preliminary exploration revealed that concentrations on the order of 200 to 500 μg. of amphetamine per cc. would probably yield useful maximum absorbancies for analytical purposes in the neighborhood of from 240 to 270 mμ. All solutions were therefore explored initially at concentrations designed to supply quantities of amphetamine within this range. In all cases, the pure solvents themselves were employed as reference liquids, and all measurements were made using the Beckman DU spectrophotometer employing 1-cm. cells.

dl-Amphetamine base was liberated from its dibasic sulfate by treatment of its aqueous solution with an excess of sodium hydroxide solution. The mixture was then extracted with ether, and the ethereal extract was washed with water and then dried over anhydrous sodium sulfate. After filtration, the ether was removed by evaporation at atmospheric pressure, after which the pressure was reduced, and the fraction distilling at 53–54° at 3 mm. Hg pressure was collected separately as pure *dl*-amphetamine. Two separate samples of the distillate showed purity rubrics of 99.46% and 99.55%, respectively, when assayed by the titrimetric method given under amphetamine in the U. S. P. XIV.

The dibasic *dl*-amphetamine sulfate employed in this investigation was dried at 105° for two hours prior to use. Assayed for its amphetamine content according to the method given under amphetamine sulfate in the U. S. P. XIV, the dried salt showed a purity rubric of 100% within the limits of the assay.

* Received March 3, 1952, from the Reed and Carnrick Laboratories, Jersey City, N. J.

Solutions for spectrophotometric examination were prepared in all cases by dissolving an accurately weighed sample of the compound under investigation in a solvent contained in a vessel calibrated to the operating temperature.

ULTRAVIOLET ABSORPTION SPECTRA

Screening explorations were first conducted on all solutions over the region from 220 mμ to about 300 mμ. More critical explorations were then made over the important absorbing region.

Figure 1 portrays the critical portion of the spectrum for solutions of different concentrations of *dl*-amphetamine base in alcohol U. S. P. The primary characteristic is a maximum absorption peak at 259 mμ, for which the specific absorbancy, $E^{1\%}_{1cm}$, calculates to 14.2. Minor secondary absorption peaks are apparent at 253 mμ and at 264 mμ. Beer's law is followed at all peaks for the concentrations employed in this study.

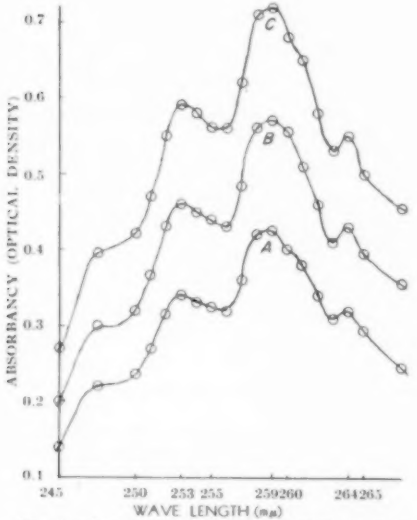


Fig. 1.—Amphetamine (base) in alcohol, U. S. P., at concentrations of: (*A*) 300, (*B*) 400, and (*C*) 500 μg. per cc.

Figure 2 portrays the critical portion of the spectrum for solutions of different concentrations of *dl*-amphetamine base in anhydrous chloroform. Maximum absorption was observed in this case at 259.5 mμ, $E^{1\%}_{1cm}$ calculated to 16.1, and Beer's law was obeyed. The minor secondary peaks observed in alcohol solution, however, were no longer evident. The one at 253 mμ had definitely flattened out into a plateau extending from 254 to 256 mμ, and the one at 264 mμ had disappeared entirely.

Figure 3 portrays the critical portion of the spectrum for solutions of different concentrations of *dl*-amphetamine base in anhydrous ether. Maximum absorption was observed here at 259 mμ, $E^{1\%}_{1cm}$ calculated to 15.4, and Beer's law was obeyed. A minor secondary peak at 254 mμ and a 1 mμ plateau commencing at 260 mμ were also observed.

Figure 4 portrays the critical portion of the spectrum for aqueous solutions of different concentrations of dibasic *dl*-amphetamine sulfate. Maximum absorption was observed at 257 mμ, and $E^{1\%}_{1\ cm}$

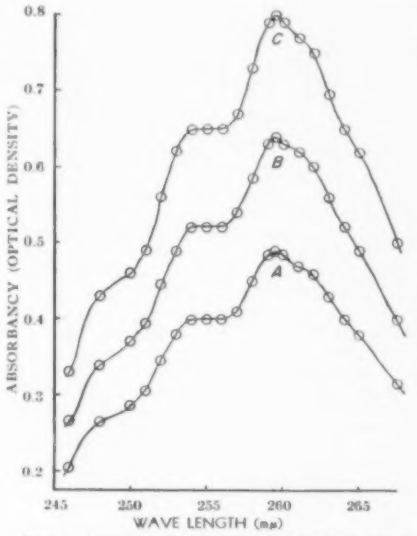


Fig. 2.—Amphetamine (base) in anhydrous chloroform at concentrations of: (*A*) 300, (*B*) 400, and (*C*) 500 μg. per cc.

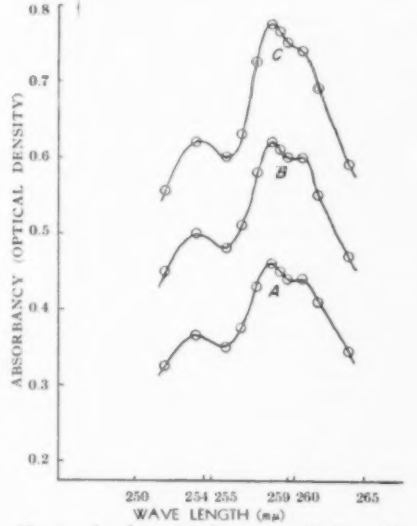


Fig. 3.—Amphetamine (base) in anhydrous ether at concentrations of: (*A*) 300, (*B*) 400, and (*C*) 500 μg. per cc.

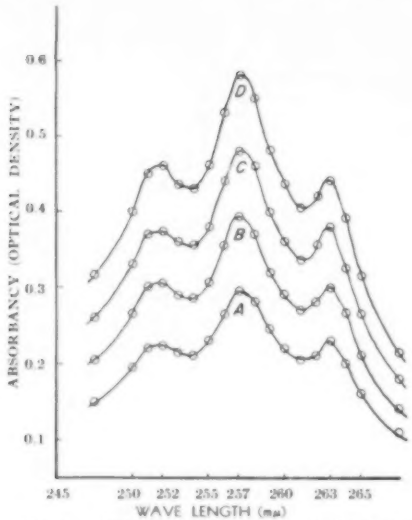


Fig. 4.—Amphetamine sulfate, U. S. P., in distilled water at concentrations of: (*A*) 300, (*B*) 400, (*C*) 500, and (*D*) 600 μg. per cc. Identical spectra are obtained when the *p*H is adjusted to 4 or 3 with addition of HCl, or when the Clark and Lubs HCl-KCl buffer mixture of *p*H 2 is employed as solvent. Solutions in 10 and 20% alcohol display the same absorption characteristics.

calculated to 9.7. Two minor secondary peaks were observed, one at 252 mμ and one at 263 mμ. Spectra for the aqueous solutions adjusted to *p*H 4, 3, and 2, and for the 10% and 20% alcohol solutions of the salt were identical with those presented here for the unaltered aqueous solution. The dibasic *dl*-phosphate was also studied under the same various conditions, and the spectra obtained were the same. This is explicable in terms of the practical equivalence of the molecular weights of the two salts; i.e., 368.49 for $(C_9H_{13}N)_2 \cdot H_2SO_4$ and 368.41 for $(C_9H_{13}N)_2 \cdot H_3PO_4$.

The *d*-forms of the free amphetamine base and of the dibasic sulfate and phosphate salts were also examined and, as was to be expected, these forms elicited absorption characteristics identical with those of the *dl*-compounds under the same conditions.

TABLE I.—SUMMARY OF USEFUL ABSORPTION DATA

Compound	Solvent	Wave Lengths at Absorbancy Peaks (mμ)	Specific Absorbancy, ($E^{1\%}_{1\ cm.}$)
dl-Amphetamine[a]	95% Alcohol	253	11.5
dl-Amphetamine[a]	95% Alcohol	259	14.2
dl-Amphetamine[a]	95% Alcohol	264	10.8
dl-Amphetamine[a]	Anh. chloroform	259.5	16.1
dl-Amphetamine[a]	Anh. ether	259	15.4
dl-Amphetamine[a]	Anh. ether	254	12.4
Dibasic *dl*[b] amphetamine sulfate[b]	Distilled water[c]	257	9.7
Dibasic *dl*-amphetamine sulfate[b]	Distilled water[c]	252	7.6
Dibasic *dl*-amphetamine sulfate[b]	Distilled water[c]	263	7.5

[a] Same data maintains for the *d*-form.
[b] Same data maintains for the *d*- and *dl*-dibasic phosphates.
[c] Same data maintains for the following solvents: distilled water adjusted to *p*H 4 or 3 with HCl; Clark and Lubs HCl-KCl buffer solution of *p*H 2, and 10% and 20% by volume of alcohol.

SUMMARY

A study has been conducted with the aim of applying ultraviolet absorption spectrophotometric analysis to the convenient determination of relatively small quantities of amphetamine (α-methylphenethylamine) in medicinal specialty products.

Specific absorbancy ($E^{1\%}_{1\ cm.}$) values have been determined for solutions of the free base in 95 per cent alcohol, in anhydrous chloroform, and in anhydrous ether, and for the dibasic sulfate and dibasic phosphate in water adjusted to various *p*H's and in 10 and 20 per cent alcohol by volume. The *d*- and *dl*-forms of amphetamine elicit identical absorption characteristics.

Through suitable conventional manipulative procedures, the method can be adapted to the analysis of a wide variety of amphetamine preparations. It has given highly satisfactory results in this laboratory.

Nitroguanylhydrazones of Aldehydes: Their Antitubercular Activity, Ultraviolet Absorption Spectra and Structure*,†

By W. D. KUMLER and PETER P. T. SAH

Some 30 nitroguanylhydrazones of aldehydes have been made. A number of these compounds show high *in vitro* antitubercular activity against *Mycobacterium tuberculosis* H37Rv. Citral and *p*-isopropyl benzaldehyde nitroguanylhydrazones showed complete inhibition of growth at concentrations of two parts per million, and *p*-nitrobenzaldehyde and *p*-dimethylaminobenzaldehyde showed partial inhibition at concentrations of two parts per hundred million. The ultraviolet absorption of the compounds has been measured. The nitroguanylhydrazones absorb at longer wave lengths and have higher extinction coefficients than the corresponding aldehydes. The nitrimine structure for the compounds is consistent with the observed spectra.

In view of the facts that thiosemicarbazones (1), guanylhydrazones, and phenylthiosemicarbazones (2) show very high power of inhibition of growth of *Mycobacterium tuberculosis* H37Rv and that the nitro group is present in the biologically active molecules of chloromycetin (3) and furacin (4), it appeared that nitroguanylhydrazones might have high biological activity against tuberculosis and other organisms. Thirty-five nitroguanylhydrazones of aldehydes have been prepared and some of them show high *in vitro* antitubercular activity. The ultraviolet spectra of the compounds have been determined and are consistent with the compounds having a nitrimine structure. The compound nitroaminoguanidine used in preparing these hydrazones has been previously used (5, 6) as a reagent for the identification of aldehydes and ketones.

EXPERIMENTAL[1]

Preparation of Nitroguanylhydrazones of Aldehydes.—Nitroaminoguanidine (0.6 Gm. or 0.005 mole) was dissolved in a mixture of solvents consisting of distilled water (20 ml.), absolute ethyl alcohol (10 ml.), and glacial acetic acid (2 ml.) in an Erlenmeyer flask (120 ml.) by warming on the steam bath. To the warm solution, a molecular equivalent in slight excess of an aldehyde which was dissolved in absolute ethyl alcohol (30 ml.), was added slowly. The reaction mixture was boiled on the steam bath for fifteen minutes. A number of nitroguanylhydrazones (those from *p*-chloro-, 2,4-dichloro-, *m*-nitro-, *p*-nitro-, *o*-hydroxy-, *p*-hydroxy-, *p*-methoxy-, and *p*-dimethylaminobenzaldehydes) precipitated out in the crystalline state, even from the hot solution. The remaining products usually separated out as beautiful crystals after standing and spontaneous cooling to room temperature. After standing for twenty-four hours, the crystals were filtered off with suction, washed with 50% ethyl alcohol, and sucked to dryness. The weight was recorded. The crystalline form was examined under the microscope. The melting point was determined, using the Thiele-Dennis tube method with di-*n*-butyl phthalate as the bath fluid. The product was then recrystallized from a suitable solvent (methyl alcohol, absolute ethyl alcohol, or a mixture of ethyl acetate and absolute ethyl alcohol). Table I shows the yields, decomposition points, crystalline forms, formula, and analysis of the nitroguanylhydrazones prepared.

The *in Vitro* Antitubercular Activity of Nitroguanylhydrazones.—These biological tests were carried out by F. G. Jones and his staff, of the Lilly Research Laboratories at Indianapolis, Ind. For the sake of convenience in comparing their activities, these nitroguanylhydrazones may be divided into the following groups:

Group I.—Derivatives from citral and *p*-isopropyl benzaldehyde. They were found to cause complete inhibition of growth of *Mycobacterium tuberculosis* H37Rv, at a concentration of 0.02 mg./10 cc. culture (2 p. p. m.) over a period of three weeks. They were inactive at higher dilution.

Group II.—Derivatives from *o*-nitrobenzaldehyde and benzaldehyde. The former caused complete inhibition of growth of *M. tuberculosis* H37Rv at a concentration of 0.2 mg./10 cc. culture over a period of three weeks. The latter caused complete inhibition of growth at the end of the first week, but at the end of the third week, a very slight growth was observed.

Group III.—Derivatives from *p*-nitrobenzaldehyde, *p*-dimethylaminobenzaldehyde, *p*-diethylaminobenzaldehyde, veratraldehyde, 2-hydroxy-5-chlorobenzaldehyde, 2-hydroxy-1-naphthaldehyde, *p*-hydroxybenzaldehyde, and cinnamaldehyde. They caused precipitation at a concentration of 0.2 mg./10 cc. culture over a period of three weeks. The first two compounds of this group showed signs of activity (partial inhibition of growth of *M. tuberculosis* H37Rv down to a concentration of 0.0002 mg./10 cc. culture. The rest were only 0.1 as active.

Group IV.—Derivatives from *o*-hydroxybenzaldehyde, *p*-methoxybenzaldehyde, *p*-chlorobenzalde-

* Received December 3, 1951, from the College of Pharmacy of the University of California, San Francisco 22, Calif.

† Paper presented before the Medicinal Section of the Twelfth International Congress of Pure and Applied Chemistry, New York City, September, 1951.

[1] Preliminary chemical preparation of the nitroguanylhydrazones has been carried out by Peter P. T. Sah and his technical staff at the Catholic University, Peiping, 1937–1944. Analyses were carried out by his technicians under the direction of Dr. C. Wang, Associate Professor of Organic Analysis, the Catholic University, Peiping, 1939–1944.

hyde, and *o*-tolualdehyde. These four compounds caused precipitation at a concentration of 0.2 mg./10 cc. culture and showed signs of power of partial inhibition of growth of *M. tuberculosis* at a concentration of 0.02 mg./10 cc. culture over a period of three weeks.

TABLE I.—YIELDS, PHYSICAL PROPERTIES, AND CHEMICAL ANALYSES OF NITROGUANYLHYDRAZONES OF ALDEHYDES

Nitroguanylhydrazones Derived from	Yield, %	Decomposition Points, ° C. (Corr.)	Appearance of Crystals	Formula	Found, %	N Calcd.
Benzaldehyde	80	197–198[d]	White, thin needles[a]	$C_8H_9O_2N_5$	33.75	33.81
			White, hairlike needles[c]		33.69	
o-Tolualdehyde	75	188–189	White needles[a]	$C_9H_{11}O_2N_5$	31.42	31.66
			White, small needles[d]		31.52	
m-Tolualdehyde	88	190–191	White needles[a]	$C_9H_{11}O_2N_5$	31.57	31.66
			White, large prisms[e]		31.72	
p-Tolualdehyde	87	189–190	White needles[a or e]	$C_9H_{11}O_2N_5$	31.59	31.66
					31.62	
p-Isopropylbenzaldehyde	88	192–193	White, short, small needles[a]	$C_{11}H_{15}O_2N_5$	27.96	28.11
			White, large prisms[c]		28.00	
o-Nitrobenzaldehyde	95	209–230	Orange-yellow prisms[b or f]	$C_8H_8O_4N_6$	33.26	33.33
					33.41	
m-Nitrobenzaldehyde	94	254–255	White, thin plates[b]	$C_8H_8O_4N_6$	33.39	33.33
			White, thin platelets[c]		33.44	
p-Nitrobenzaldehyde	99	245–246	Yellow, fine needles[b or c]	$C_8H_8O_4N_6$	33.50	33.33
					33.47	
o-Chlorobenzaldehyde	91	206–207	White, narrow plates[a]	$C_8H_8O_2N_5Cl$	14.72[h]	14.67
			White, stout needles[e]			
p-Chlorobenzaldehyde	100	218–219	White, fine needles[b]	$C_8H_8O_2N_5Cl$	14.63	14.67
			White, small needles[c]		14.60	
2,4-Dichlorobenzaldehyde	90	227–228	White, fine needles[b]	$C_8H_7O_2N_5Cl_2$	25.85	25.70
			Pale-yellow rods and prisms[c]		25.82	
3,4-Dichlorobenzaldehyde	58	230–231	White, small needles[b]	$C_8H_7O_2N_5Cl_2$	25.92	25.70
			White, small prisms[e]		25.78	
o-Hydroxybenzaldehyde	99	229–230[g]	Pale-yellow, long needles[b]	$C_8H_9O_3N_5$	31.43	31.38
			Pale-yellow, small needles[e]		31.29	
p-Hydroxybenzaldehyde	99	249–250	Pale-yellow needles[b,c, or e]	$C_8H_9O_3N_5$	31.32	31.38
					31.27	
o-Methoxybenzaldehyde	98	199–200	White, shining, narrow plates[b]	$C_9H_{11}O_3N_5$	29.45	29.53
			White rods and thick plates[e]		29.39	
p-Methoxybenzaldehyde	100	210–211	White, long, stout needles[b]	$C_9H_{11}O_3N_5$	29.49	29.53
			White needles[c]		29.56	
o-Ethoxybenzaldehyde	93	197–198	White, shining, prismatic needles[b]	$C_9H_{11}O_3N_5$	27.76	27.88
			White, shining prisms[e]		27.93	
p-Dimethylaminobenzaldehyde	100	203–204	Pale-yellow needles[b]	$C_{10}H_{14}O_2N_6$	33.63	33.59
			Yellow, small needles[c or e]		33.52	
p-Diethylaminobenzaldehyde	93	188–189	Yellow needles[b]	$C_{12}H_{18}O_2N_6$	30.15	30.20
			Yellow, shining prisms[c]		29.98	
Vanillin	96	197–198[k]	Pale-yellow, small needles[b]	$C_9H_{11}O_4N_5$	27.58	27.66
			Pale-yellow, fine needles[d]		27.52	
Piperonal	100	227–228[l]	White rods and thick plates[b]	$C_9H_9O_4N_5$	27.91	27.88
			White rods[c or e]		27.79	
Veratraldehyde	100	209[m]	Pale-yellow needles[b]	$C_{10}H_{13}O_4N_5$	26.15	26.21
			Pale-yellow, fine needles[c]		26.33	
Cinnamaldehyde	99	195[n]	White granules[b]	$C_{10}H_{11}O_2N_5$	29.87	30.03
			White crystalline powder[c]		30.11	
Hydrocinnamaldehyde	45	126	White needles and rods[b]	$C_{10}H_{13}O_2N_5$	29.64	29.77
			White needles[c]		29.59	
2-Hydroxy-1-naphthaldehyde	95	250–251	Greenish-yellow platelets[b]	$C_{12}H_{11}O_3N_5$	25.55	25.64
			Greenish-yellow crystalline powder[e]		25.59	
2-Hydroxy-3-methoxybenzaldehyde	95	214–215	Pale-yellow prisms[b]	$C_9H_{11}O_4N_5$	27.49	27.66
			Pale-yellow rods and prismatic needles[c]		27.54	
2,3-Dimethoxybenzaldehyde	76.4	198–199	White needles[b or e]	$C_{10}H_{13}O_4N_5$	26.25	26.21
					26.28	
2,4-Dihydroxybenzaldehyde	100	262–263	Yellow prisms[b or e]	$C_8H_9O_4N_5$	29.79	29.83
					29.88	
3-Ethoxy-4-hydroxybenzaldehyde	95	200–201	Pale-yellow crystalline powder[b]	$C_{10}H_{13}O_4N_5$	25.99	26.21
			Pale-yellow, prismatic needles[c]		26.03	

Continued on page 377

TABLE I (*Continued from page 376*)

Nitroguanylhydrazones Derived from	Yield, %	Decomposition Points, ° C. (Corr.)	Appearance of Crystals	Formula	Found, %	N[g] Calcd.
3,4-Diethoxybenzaldehyde	95	206–207	Very pale-yellow, fine needles[b, c]	$C_{12}H_{17}O_4N_5$	23.66 23.59	23.72
α-*n*-Amylcinnamaldehyde	98	172–173 (slow dec.)	White, thin plates[b] White needles[e]	$C_{15}H_{21}O_2N_5$	23.51 23.47	23.56
Furfural	99	220–224	Yellow hairlike needles[b] Pale-yellow needles[e]	$C_6H_7O_3N_5$	35.35 35.46	35.53
5-Nitrofurfural	95	249–250	Golden yellow needles[b] Yellow needles[f]	$C_6H_6O_5N_6$	34.66 34.83	34.71
Citral	82	141–142	Pale-yellow, shining plates[b] Pale-yellow prisms[d]	$C_{11}H_{19}O_2N_5$	27.58 27.50	27.65
2-Hydroxy-5-chlorobenzaldehyde	94	228–229	Pale-yellow needles[b or c]	$C_9H_8O_2N_5Cl$	13.68[h] 13.82	13.76

[a] From 50% EtOH; [b] 66% EtOH; [c] ab. EtOH; [d] 50% MeOH; [e] ab. MeOH; [f] ab. EtOH + CH_3COOEt; [g] modified Dumas method; [h] % Cl by Stepanow method; [i] m. p. reported by Whitmore, Revukas, and Smith, 188°; [j] m. p. reported by Whitmore, *et al.*, 213°; [k] m. p. reported by Whitmore, *et al.*, 186–187°; [l] m. p. reported by Whitmore, *et al.*, 220°; [m] m. p. reported by Smith and Shoub, 195°; [n] m. p. reported by Whitmore, *et al.*, 186°.

Ultraviolet Absorption Spectra.—The ultraviolet light absorption of these compounds is characterized by a band of high extinction coefficients (ε of 23,000 to 55,100) at long wave lengths (λ of 312 to 348 mμ), and in most cases one, and in some instances two, additional bands of lesser extinction coefficients (ε of 6,680 to 19,600) at shorter wave lengths (λ of 224 to 300 mμ). The extinction coefficient maxima and wave-length maxima for the various bands of these compounds are given in Table II.

The effect on the spectrum of converting the various aldehydes to the nitroguanylhydrazones is to cause the long wave-length band to move to longer wave lengths and bring about a large increase in its extinction coefficient. This is illustrated in Table III.

This bathochromic and hyperchromic effect is the usual result of an increase in conjugation of a system. It is then pertinent to examine the various structures the compounds might have in order to see if they are compatible with the observed change in spectra.

The structure of the parent compound nitroguanylhydrazone used in making these compounds has been a controversial subject for a number of years.

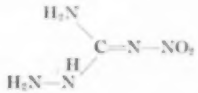
TABLE II.—THE EXTINCTION COEFFICIENT MAXIMA AND WAVE-LENGTH MAXIMA FOR THE VARIOUS BANDS OF NITROGUANYLHYDRAZONES OF ALDEHYDES

Nitroguanylhydrazones of the Following Aldehydes	λ	ε	λ′	ε′	λ″	ε″	$\frac{\lambda}{\lambda'}$	$\frac{\lambda'}{\lambda''}$
Benzaldehyde	315	32,600	...	....	...	....	..	..
p-Hydroxybenzaldehyde	334	32,000	255	8,870	228	12,000	1.31	1.46
Cinnamaldehyde	338	55,100	256	10,300	232	12,400	1.32	1.46
α-*n*-Amylcinnamaldehyde	332	42,900	256	8,830	...	....	1.25	..
o-Tolualdehyde	318	33,500	250	7,700	...	....	1.27	..
m-Tolualdehyde	319	33,400	...	....	...	....	..	..
p-Tolualdehyde	318	34,300	249	6,900	224	10,200	1.28	1.42
Salicylaldehyde	341	27,200	252	6,680	...	....	1.35	..
o-Methoxybenzaldehyde	338	46,700	254	8,720	...	....	1.33	..
p-Methoxybenzaldehyde	332	37,700	255	8,750	...	....	1.30	..
2-Hydroxy-3-methoxybenzaldehyde	323	28,300	254	6,470	...	....	1.27	..
4-Hydroxy-3-methoxybenzaldehyde (vanillin)	342	35,000	262	9,500	...	....	1.32	..
3,4-Dimethoxybenzaldehyde	338	37,500	256	9,910	...	....	1.32	..
o-Ethoxybenzaldehyde	340	30,400	...	....	...	....	..	..
4-Hydroxy-3-ethoxybenzaldehyde	342	32,700	260	10,050	...	....	1.32	..
3,4-Diethoxybenzaldehyde	342	37,000	259	10,300	...	....	1.32	..
o-Chlorobenzaldehyde	322	36,900	...	....	...	....	..	..
p-Chlorobenzaldehyde	321	35,400	...	....	...	....	..	..
2-Hydroxy-5-chlorobenzaldehyde	348	24,800	300	19,600	...	....	1.16	..
2,4-Dichlorobenzaldehyde	325	37,400	...	....	...	....	..	..
3,4-Dichlorobenzaldehyde	321	29,600	265	12,000	...	....	1.21	..
o-Nitrobenzaldehyde	305	23,000	...	....	...	....	..	..
m-Nitrobenzaldehyde	312	31,300	...	....	...	....	..	..
p-Nitrobenzaldehyde	341	29,200	...	....	...	....	..	..
p-Dimethylaminobenzaldehyde	374	42,400	273	13,000	248	11,450	1.37	1.51
p-Diethylaminobenzaldehyde	380	40,500	275	12,700	249	10,140	1.38	1.53
p-Isopropylbenzaldehyde	322	35,600	...	....	...	....	..	..
Citral	314	33,400	...	....	...	....	..	..
Furfural	326	36,600	254	7,610	...	....	1.28	..
5-Nitrofurfural	370	24,500	293	13,850	233	7,280	1.26	1.59
Piperonal	342	34,100	260	9,100	...	....	1.31	..

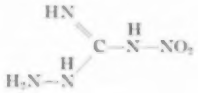
TABLE III.—THE EFFECT ON THE SPECTRUM OF CONVERTING THE VARIOUS ALDEHYDES TO THE NITROGUANYLHYDRAZONES

	λ	ε
Piperonal	313	9,120
Piperonal nitroguanylhydrazone	342	34,100
Vanillin	310	10,400
Vanillin nitroguanylhydrazone	342	35,000
p-Nitrobenzaldehyde	265	11,500
p-Nitrobenzaldehyde nitroguanylhydrazone	341	29,200
2-Hydroxy-5-chlorobenzaldehyde	338	3,720
2-Hydroxy-5-chlorobenzaldehyde nitroguanylhydrazone	348	24,800

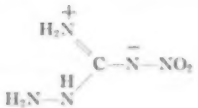
Recently evidence has been presented that nitroguanidine (7) and nitroaminoguanidine (8) are nitrimines



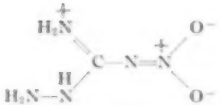
and not nitramines



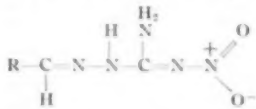
or zwitterions



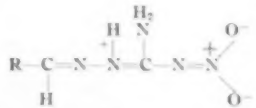
or



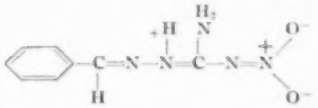
The hydrazones formed from the compound would be expected to have a similar structure, namely:



It will be noticed that this structure has a resonating form

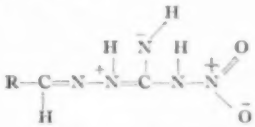


which enables the conjugation to extend clear across the molecule. If R is a benzene ring as is the case with most of our compounds, this also is conjugated with the rest of the molecule in this form with a separation of charge:

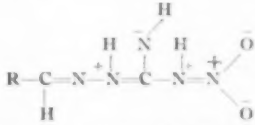


The effect of the contribution of this form on the spectrum will be bathochromic and hyperchromic. Any effect that lowers the energy of the excited state compared to the ground state will shift the long wave-length band to longer wave length (bathochromic effect) (9). The form with a separation of charge makes a greater contribution to the excited state than to the ground state. Since the conjugation is greater in the excited state than in the ground state, the effect of such conjugation will be to lower the energy of the excited state with respect to the ground state and thus produce the observed bathochromic shift. The lowering of the energy of the form with separation of charge by the extra conjugation will increase the resonance energy involving this form. The extra stability and the high polarity of the excited state are associated with a higher extinction coefficient, which depends upon several factors including the polarity of the excited states, the probability of transition from the ground to the excited state, and the change in dipole in going from the ground to the excited state.

The nitrimine structure with its attendant contribution from a stabilized form with a separation of charge adequately accounts for the ultraviolet absorption of these compounds. On the other hand, the nitramine structure does not afford an opportunity for stabilizing the excited state to any such extent, for the form



is much less stable because of the negatively charged nitrogen and the lesser amount of conjugation, while the form



is highly unstable due to the like charge on adjacent atoms, the three separations of charge, and the negatively charged nitrogen. The contribution of both of these forms would be so small as to be negligible.

CONCLUSIONS

Some 35 nitroguanylhydrazones of aldehydes have been described. A number of these compounds show very high activity *in vitro* against *Mycobacterium tuberculosis* H37Rv. The ultraviolet spectra of 31 of the nitroguanylhydrazones have been measured and are consistent with the compounds having a nitrimine rather than a nitramine structure.

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Khellin. Part III. Polarographic Determination of Khellin in Serum*

By M. M. BURKE, T. L. FLANAGAN, R. L. YOUNG, S. D. BAILEY, and A. E. HEMING

A polarographic assay for khellin in serum has been developed. The method has been applied to *in vivo* studies and has been found adequate to show the presence of khellin in small amounts (0–10 μg./ml.). The average recovery of khellin was 90 per cent, the precision of the method varying with the blood level. It was ±5 per cent at a level of 10 μg./ml. and was ±40 per cent at 1 μg./ml.

TWO METHODS have been used for the determination of khellin in blood (1, 2), namely, the alkaline hydrolysis colorimetric method originally devised by Fahmy and El-Keiy (3), subsequently modified (1, 2, 4, 5), and the biological method utilizing the rectal caecum of the fowl (1, 2, 4, 5).

Barsoum, Kenawy, and El-Sheehy (1) studied the concentration of khellin in blood of both dog and human subjects following administration by various routes. Our interest in the extension of these studies led to attempts to assay khellin in serum. In our laboratories the colorimetric method used by the previous workers gave unreliable results (6). Similarly, the biological method was evaluated by associates (7), but it could not be adapted readily to a quantitative procedure.

A colorimetric khellin assay utilizing the color formed on reaction between khellin and sulfuric acid has since been reported (8). Although it was found to be superior to the alkali test, an attempt to adapt this method to blood level studies was unsuccessful, since the sensitivity was not great enough to give reliable results with the small amounts of khellin present in the serum.

This paper presents a new assay for khellin in serum based on its polarographic reduction, which has been previously reported (6, 9). By this means, very small amounts of khellin in serum can be detected reliably. Following oral or intravenous administration, the khellin serum levels of seven dogs over a period of twenty-four hours showed a consistent absorption pattern.

PROCEDURE

Processing of Serum.—Two milliliters of serum was delivered into a 50-ml. centrifuge tube and 25 ml. of redistilled 95% ethanol was added with agitation. After fifteen minutes, the suspension was centrifuged until clear and the supernatant liquid was decanted into another centrifuge tube. The precipitate was washed twice with 5-ml. portions of alcohol, the suspension was centrifuged as before, and the protein precipitate was discarded. Then the combined filtrate and washings were evaporated in a current of air on a water bath at 60° to a volume of 3 ml. After the walls of the tube were washed down with 3–5 ml. of alcohol, evaporation was resumed until the total volume was again about 3 ml. To obtain additional precipitation of proteins, the tube was stoppered and refrigerated for at least one-half hour in the freezing compartment of a standard refrigerator. The suspension was again centrifuged and the clear supernatant liquid decanted into a 125-ml. separatory funnel, the stopcock of which was lubricated with distilled water. The precipitate in the centrifuge tube was washed with 4 ml. of ethanol cooled to 5°, the suspension centrifuged, and the washings were combined with the decantate in the separatory funnel.

Eighty milliliters of water was added to the ethanolic concentrate in the separatory funnel and

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three extractions were made with 10, 7, and 6 ml. of redistilled chloroform, respectively. The mixture was shaken by gentle inversion of the funnel for three minutes. At least twenty minutes was allowed for clear separation of the layers. With each extraction the chloroform layer was drawn off into a 10-ml. glass-stoppered Erlenmeyer flask, and evaporated on a steam bath at 60° in a current of air.

After the combined chloroform extracts had been evaporated to dryness, the sides of the flask were washed down with a small volume of chloroform and the evaporation continued, the flask being tilted so that the residue formed in a small spot.

Polarographic Procedure.—To the flask was added 0.60 ml. of redistilled water and 1.0 ml. of a solution of crystalline khellin containing 40 μg./ml. The addition of khellin was made in order to raise the total concentration of khellin to the more reliable range of the calibration curve. The flask was stoppered and heated gently over a steam bath to facilitate solution of the khellin from the residue. After cooling, the stopper was removed, the residue broken up and spread with a glass rod to prevent occlusion of khellin in the spot residue. Then the flask was restoppered and reheated on a steam bath. After cooling, the solution was filtered through a medium porosity sintered-glass microfunnel. An aliquot (1.0 ml.) was transferred to an electrolysis cell of 2-ml. capacity, and 0.25 ml. of a 0.1 *N* tetraethylammonium hydroxide solution was added to the aliquot. This medium was used despite its alkalinity because the khellin wave in this supporting electrolyte had been previously found to be higher with a wider plateau than the wave in various other media tested. Nitrogen was bubbled through the solution in the cell for ten minutes and then over the solution while the polarogram was being recorded on a Sargent-Heyrovsky Model XII polarograph at a sensitivity of 2, (0.010 μa./mm.) covering the voltage range from −0.9 to −2.0 volts. The half-wave potential was approximately −1.5 volts *vs.* the S.C.E. Timing is of importance here to prevent decomposition of the khellin in the basic medium. No difficulty was encountered with a series of samples when the time of starting the polarogram varied from eleven to fourteen minutes after the addition of the base.

In the foregoing preparation of samples for measurement in the polarograph, all aliquots and dilutions have been selected, and the calibration curve (Fig. 1) has been constructed so that the concentration of khellin in the original serum sample can be read directly from the curve.

Animal Study.—The dogs used in the absorption study were fasted for at least eighteen hours before administration of the drug and during the period of observation. At stated intervals a small sample of blood was taken from the jugular vein, allowed to clot, then centrifuged until the serum was clear. The serum was processed as outlined above.

RESULTS

The calibration curve for serum khellin concentration *versus* polarographic response (Fig. 1) was based on the data obtained from 11 serum blanks processed in the same manner as reported for the *in vivo* samples, except for the addition of varying amounts of khellin to the final solution. The blanks were obtained from eight different dogs, and no interfering irregularities occurred in the polarograms.

In Fig. 2, "*a*" shows a background curve obtained with a blank serum sample processed as outlined above, "*b*" shows a typical polarogram obtained in the *in vivo* studies, and "*c*" shows a different polarogram of the series to illustrate the method of measuring step height.

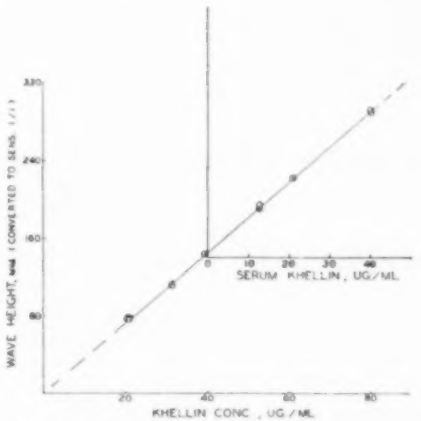

Fig. 1.—Polarographic khellin curve in processed serum (11 determinations). Supporting electrolyte 0.025 *N* tetraethylammonium hydroxide, temp. 25.0°, capillary constant 1.731 mg. 2/3 sec. $^{-1/2}$.

In Table I are summarized percentages of khellin recovered from serum for a series of samples of serum to which khellin was added prior to processing in the identical manner as was done for the *in vivo* studies. The average recovery value obtained, as determined from the calibration curve of Fig. 1, was 90%.

TABLE I.—RECOVERY OF KNOWN AMOUNTS OF KHELLIN FROM SERUM

Khellin Added; μg./Ml.	Khellin Found, μg./Ml.	Recovery, %
20.6	18.5	90
20.6	18.7	91
31.3	26.6	85
31.3	26.9	86
39.2	35.7	91
50.4	45.4	90
52.7	49.5	94
52.7	46.9	89
61.0	61.0	100
80.1	69.7	87
80.1	74.5	93
	Average	90

Studies were made *in vivo* on seven dogs, one of which was given khellin intravenously (5 mg./Kg.), while the others were given the drug orally (9–10 mg./kg.). In Fig. 3 (curve *A*) are shown typical values for the blood levels found in dogs which had

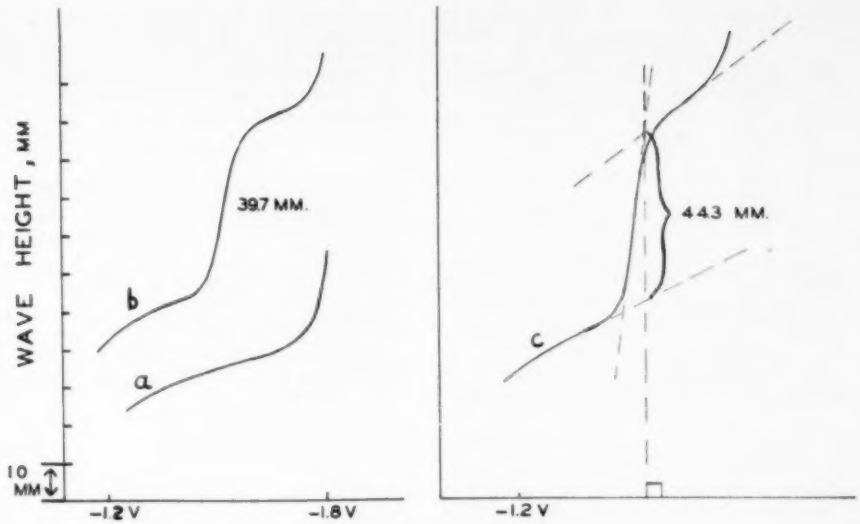

Fig. 2.—Representative polarograms, sens. 1/3. *a*, Serum blank. *b*, Serum khellin polarogram. *c*, Serum khellin polarogram illustrating the method of measuring the step height.

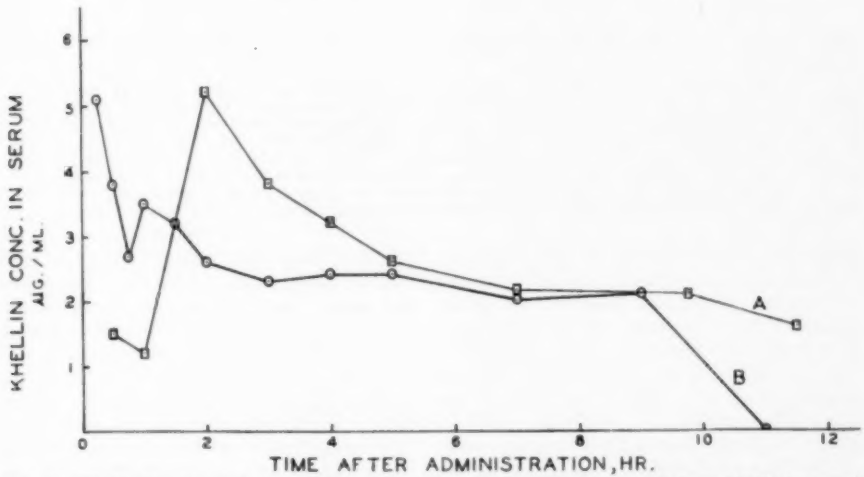

Fig. 3.—Khellin blood level study. *A*, Oral administration, dog 9.0 Kg., dosage 80.2 mg. khellin by mouth *B*, Intravenous administration, dog 17.6 Kg., dosage 5.0 mg./Kg. (5% in 40% aqueous sodium benzoate)

been given an oral dose. The data show very little khellin in the serum one-half hour after administration of the drug. The maximum serum level was reached consistently at two to three hours after ingestion and showed a gradual tapering off until no appreciable amounts of khellin were detected after twenty-four hours. At the lowest level observed (1.2 μg./ml.) the precision, based on a 2% variation in step height, was ±40%. At the highest level (10 μg./ml.), the precision was ±5%. The maximum khellin concentration in the serum varied from 5 to 10 μg./ml.

Curve *B* in Fig. 3 shows the blood level of khellin following a single intravenous injection of 5 mg./Kg. There was a rapid initial decrease of khellin concentration during the first two hours, followed by a very slow decline for about nine hours. Little or no khellin was found after twenty-four hours.

TABLE II.—COMPARISON OF KHELLIN LEVELS OBTAINED BY ALKALINE HYDROLYSIS AND POLAROGRAPHIC METHODS

Time After Injection, Min.	Alkaline Hydrolysis,[a, b] μg./Ml.	Polarographic,[c, d] μg./Ml.
10	22	...
15	..	5
20	13	...
30	..	4
40	9	...
45	..	3
60	8	3.5

[a] Data on whole blood from Barsoum, *et al.* (1), who have demonstrated that khellin is uniformly distributed between cells and plasma.
[b] Dog received 5.8 mg./Kg. of khellin intravenously.
[c] Serum levels.
[d] Dog received 5.0 mg./Kg. of khellin intravenously.

No studies of blood levels following true oral administration of khellin have been previously reported. However, a preliminary comparison of our intravenous experiment with the results on whole blood reported by Barsoum, *et al.* (1), would suggest that the present method may yield somewhat lower results than the alkaline hydrolysis method (for comparison see Table II).

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Nitrogen Tetroxide Studies. I. The Preliminary Oxidation of L-Sorbose*,†

By OLAV BRAENDEN and OLE GISVOLD‡

Using essentially anhydrous conditions, L-sorbose was oxidized by nitrogen tetroxide dissolved in carbon tetrachloride. The oxidized sugar yielded an alcohol-insoluble calcium salt whose behavior and properties resembled those of the calcium salt prepared from an authentic sample of methyl-2-keto-gulonate. Optimal conditions were achieved under which maximum yields were obtained of an acid that upon lactonization had dye-reducing properties and led to the isolation of crystalline ascorbic acid. Thus it was assumed that one of the sugar acids resulting from the anhydrous oxidation of L-sorbose by nitrogen tetroxide was 2-keto-gulonic acid.

NITROGEN TETROXIDE has been used to oxidize selectively the primary alcohol groups of the glucose residues of cellulose to carboxyl groups (1). This oxidation was carried out either in a gaseous phase or using carbon tetrachloride as a solvent. A small amount of nitric acid was usually added and no attempt was made to keep the reaction mixture dry. In fact, the addition of nitric acid was proposed as a catalyst. Even under these conditions, the secondary alcohol groups apparently were not attacked. It is well known that all carbohydrates have lactal structures. Common monosaccharides, both in the solid state and in solution, exist chiefly in the pyranose form. In aqueous solution this modification is not stable, as evidenced by the phenomenon of mutarotation. On the other hand, in the solid dry state either the α- or β-modification can be kept for long periods of time. This would lead one to believe that the pyranose ring remains intact under these conditions. Therefore, if a suitable oxidizing agent were available that would selectively attack primary alcohol groups under essentially anhydrous conditions, one could oxidize the primary alcohol of the monosaccharides to yield the corresponding acid. In the case of the aldoses, the uronic acids would be obtained, whereas the 2-ketoses would yield the 2-keto "onic" acids. 2-Ketogulonic acid is of particular interest because it is the immediate precursor of ascorbic acid.

The production of 2-ketogulonic acid via the direct oxidation of L-sorbose would constitute a one-step reaction that would have both theoreti-

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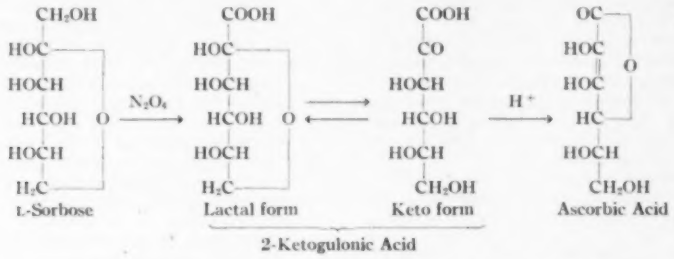
cal and practical interest. The oxidation of L-sorbose by means of nitrogen tetroxide under anhydrous conditions has been accomplished. 2-Ketogulonic acid was not isolated, however. L-Ascorbic acid was isolated from the oxidized L-sorbose after the latter had been lactonized.



EXPERIMENTAL

In order to keep the reaction mixture dry, Drierite was chosen because it is inert in character, very efficient, and acts very rapidly. In order to improve its usefulness still further, it was reduced to a powder. The sorbose used was obtained from Chas. Pfizer & Co., New York. It was not subjected to further purification; however, it was dried in a vacuum desiccator over calcium chloride for several days. The rotation was $[\alpha]^{30}_D = -43.1$ as compared to the reported rotation (2) of -42.9. In some of the preliminary experiments, nitrogen textroxide was prepared by heating lead nitrate (3) and condensing the resulting vapors. However, this was a tedious and somewhat uncertain procedure. Therefore, nitrogen tetroxide was purchased in small, readily-available tanks (The Matheson Co., Joliet, Ill.). For convenience of manipulation and purification, the gas was allowed to escape from the tank into an Erlenmeyer flask that was chilled in a Dewar flask by means of dry ice. A redistillation was effected at room temperature under a light vacuum, using essentially the same condensing technique. The resultant solid could be stored in the refrigerator and used as needed. The carbon tetrachioride was distilled over Drierite to remove any water that might possibly be present. Filter-Cel (Johns-Manville & Co.) was dried in the oven at 150° for twenty-four hours and subsequently was stored in a vacuum desiccator to be used as needed.

A number of preliminary experiments definitely indicated that, after oxidation and lactonization, a substance was obtained that strongly reduced 2,6-dichlorophenol-indophenol, a property that is shared with ascorbic acid and related compounds. The amounts of reactants and inert ingredients were varied in order to obtain the maximum yields. The results of a number of experiments, therefore, enabled the formulation of the following procedure: 9 Gm. of sorbose (1/20 mole), 27 Gm. of Drierite, and 9 Gm. of dry Filter-Cel, finely powdered in a stone mill, were placed in a 250-cc. Erlenmeyer flask to which was added 23.4 Gm. of nitrogen tetroxide dissolved in 50 cc. of carbon tetrachloride. The Filter-Cel was added to facilitate a smooth dispersion of the sorbose and also to help prevent clumping. The reaction vessel was stoppered with a calcium chloride drying tube to allow the escape of vapors and prevent the exchange of moist air. The mixture was allowed to stand for forty-eight hours at room temperature, with occasional shaking. During the initial stages of the reaction, the temperature of the reaction mixture rose several degrees. The color shifted from brown to greenish-brown, which indicated that some nitrogen trioxide had been formed (4). The excess nitrogen oxide and carbon tetrachloride were removed from the reaction mixture by means of a suction funnel, and dried air was passed through the mixture until it appeared dry and white. The solid mixture was extracted with 200 cc. of methanol. Hydrogen sulfide gas was passed into the alcoholic solution in order to remove traces of nitric acid that might be present, as such traces had previously been shown to be very undesirable during subsequent manipulations. After the alcoholic solution had stood for about twelve hours, it was filtered to free it from any sulfur that might be present. Distilled water (50 cc.) was added, and the alcohol was removed under vacuum until a volume of about 40 cc. was obtained. Calcium carbonate was then added in excess, and the excess calcium carbonate was filtered off and washed with enough water to obtain 50 cc. of a clear filtrate. Methyl alcohol (200 cc.) was added to this aqueous filtrate in order to precipitate the calcium salts of 2-ketogulonic acid, and possibly other sugar acids, that might have been obtained. This precipitate was washed with 50 cc. of methyl alcohol and subsequently dried. The average yield of calcium salts was 8.3 Gm. The sugar acids were liberated from their calcium salts by using stoichiometric amounts of oxalic or sulfuric acids. The resultant product gave an amorphous mass from which no crystalline material could be obtained. Methylation with diazomethane in absolute methanol and ether also gave an amorphous product from which crystalline material was unobtainable. Nevertheless, these products when heated in the presence of aqueous carbonic, hydrochloric, or sulfuric acids gave a solution which very strongly reduced 2,6-dichlorophenol-indophenol. The calcium salt was analyzed with the following results:

Anal.—Calcd. for $Ca(C_6H_9O_7)_2$: C, 33.8; H, 4.26. Found: C, 34.34, 33.50; H, 4.41, 4.60.

An authentic sample of calcium-2-ketogulonate from an authentic sample of methyl-2-ketogulonate (obtained through the courtesy of Chas. Pfizer & Co., New York) was prepared as follows: 10 Gm. of the methyl ester was dissolved in 50 cc. of water without heating, and 5 Gm. of dry calcium hydroxide was

added. The mixture was stirred for one hour and filtered. Methanol (200 cc.) was added to the clear filtrate, and the white precipitate that formed was filtered and washed with methanol. It was then dried in a vacuum desiccator to constant weight. The yield was 9 Gm.

Anal.—Calcd. for $Ca(C_6H_9O_7)_2$: C, 33.80; H, 4.26. Found: C, 34.46; H, 4.73.

A quinine salt was prepared by saturating an aqueous solution of the sugar acids with quinine base. After concentration *in vacuo* and several days of standing at room temperature, small needle-shaped crystals were obtained that melted at 155–157°. No depression in melting point could be observed when a mixed melting point was taken with a quinine salt prepared from an authentic sample of 2-ketogulonic acid.

Table I was abstracted from 47 experiments that were performed in order to obtain the maximum yields of the calcium salts of the sugar acids.

When amounts less than 9.2 Gm. of nitrogen tetroxide were used for a period of forty-eight hours or less, no detectable amounts of calcium salts could be observed.

TABLE I

Sorbose, Gm.	N_2O_4, Gm.	Time of Oxidation, Hr.	Calcium Salt Formed, Gm.	2-Ketogulonic Acid, % $\frac{a \times 100}{b}$
9	9.2	48	0.4	...
9	11.5	36	1.2	53.3
9	13.8	24	2.8	55.7
9	13.8	36	3.2	54.1
9	16.1	36	4.6	52.2
9	18.4	24	6.1	52.9
9	18.4	48	7.3	58.8
9	23.0	12	5.6	53.3
9	23.0	48	8.3	58.0

Estimation of the Amounts of Reducing Substances Obtained upon Lactonization of the Calcium Salts of the Sugar Acids.—In the preceding studies the total sugar acids were conveniently isolated as their calcium salts. The quantitative resolution of the sugar acids presented a serious problem in these preliminary studies. It was hoped that some insight as to the amount of 2-keto-sugar acids could be gained by subjecting the total calcium salts to the action of an acid in order to effect lactonization under standardized conditions. In the case of the 2-keto-sugar acids, the amount of lactonization could be measured quantitatively by titration with a standard iodine solution. Because a relatively large number of techniques have been reported in the literature involving the use of a number of acids of varying strengths and other conditions, the following procedure was arbitrarily adopted. A reference sample of supposedly pure calcium 2-keto-gulonate was used for comparison purposes. Samples of exactly one gram of the calcium salt from selected experiments were placed in identical test tubes and dissolved in 5 cc. of water, and 5 cc. of 40% sulfuric acid was added. For comparison, 1 Gm. of the reference calcium 2-keto-gulonate was treated in the same way. The mixtures were heated for twenty minutes in a water bath (5). The test tubes were then immediately cooled under the tap and the contents were assayed for reducing properties as described by the U. S. P. XIV for the quantitative determination of ascorbic acid. The contents of each tube were transferred to a 250-cc. Erlenmeyer flask, and 115 cc. of water was added in order to dilute the sulfuric acid to the same strength as that described in the U. S. P. XIV. The samples were then titrated with 0.1 *N* iodine solution, using starch T. S. as an indicator.

By comparing the amounts of 0.1 *N* iodine solution consumed in the unknown with the reference material, an approximate estimation of the 2-keto-gulonic acid content of the sample was obtained by substituting the values found by these determinations in the following formula: $\frac{a \times 100}{b}$, where

a = number of cc. of 0.1 *N* iodine consumed by 1 Gm. of pure calcium-2-keto-gulonate when both were treated as described above. The last column of Table I expresses the results obtained using the assay described.

Preparation of Ascorbic Acid.—In order to establish that 2-keto-L-gulonic acid was actually being formed as a result of the oxidation of L-sorbose with nitrogen tetroxide, the following experiment was performed: 20 Gm. of the calcium salt was treated with a stoichiometric amount of oxalic acid in 200 cc. of water. After removal of the calcium oxalate, the solution was heated on a water bath under a constant stream of carbon dioxide (6) for two hours. The solution was concentrated to a thick syrup *in vacuo* and mixed with acetone. The acetone solution was filtered and the filtrate concentrated *in vacuo* to a syrup, which, upon standing in the ice chest, gave small needle-shaped crystals. This mixture was carefully diluted with a few cubic centimeters of ice-cold acetone and then was filtered by suction through a chilled glass filter. The crystals were washed with a small amount of ice-cold acetone and subsequently were recrystallized twice from methanol. They melted at 188–189° and, when mixed with pure ascorbic acid, showed no change in melting point.

Anal.—Calcd. for $C_6H_8O_6$: C, 40.91; H, 4.58. Found: C, 41.20; H, 4.99.

Attempted Oxidation of L-Sorbose by Nitrogen Tetroxide Using Nitric Acid as a Catalyst.—When a small amount of nitric acid was added to the oxidation mixture according to Kenyon's procedure (1), only a small amount of reducing substances (2,6-dichlorophenol-indophenol) was obtained, together with a large amount of oxalic acid which was authentically characterized.

Attempted Oxidation of L-Sorbose by Gaseous Nitrogen Tetroxide.—An apparatus was designed in which dry sorbose, Drierite, and Filter-Cel were rendered porous to the vapors of nitrogen tetroxide. Little, if any, reducing substances (2,6-dichlorophenol-indophenol) were obtained when the reaction mixture was worked up in the usual manner previously described. According to Kenyon (1), cellulose can be oxidized to carboxy cellulose by gaseous nitrogen tetroxide in the presence of a little nitric acid.

Oxidation of the Methyl Glycoside Sorbose.—Methyl-L-sorbopyranoside was prepared according to Arragon (7). Nine grams of dry methyl sorbopyranoside was pulverized with 27 Gm. of Drierite and 9 Gm. of Filter-Cel. It was then oxidized with a mixture of 23 Gm. of nitrogen tetroxide and 50 cc. of carbon tetrachloride for forty-eight hours at room

temperature. The reaction mixture was then worked up as previously described and 8.2 Gm. of a calcium salt was obtained. The calcium salt, upon treatment with acid, exhibited strong reducing (2,6-dichlorophenol-indophenol) properties. One-gram samples, when analyzed for their reducing properties, consumed an average of 14.1 cc. of 0.1 *N* iodine, which corresponds to about 55.4% 2-keto-gulonic acid.

Preliminary Qualitative Testing of the Oxidation of Sucrose with Nitrogen Tetroxide.—Ten grams of dried sucrose was pulverized with 80 Gm. of Drierite and 10 Gm. of Filter-Cel. The mixture was treated with 50 Gm. of nitrogen tetroxide in 100 cc. of carbon tetrachloride for a period of forty-eight hours at room temperature. The reaction mixture was then worked up as previously described. The methyl alcohol solution was concentrated *in vacuo* to about 50 cc. When ether was added to this concentrate, a precipitate was formed which was filtered off with suction and dried in a vacuum desiccator. The resulting solid was acidic and showed very strong reducing (2,6-dichlorophenol-indophenol) properties after it had been heated with strong acids. One might tentatively infer that this reduction is due to oxidation of the carbon atom in the 1-position of the fructose residue to a carboxyl group, followed by lactonization by acid treatment to give the enediol structure found in ascorbic acid.

SUMMARY

L-Sorbose has been oxidized under anhydrous conditions to give good yields of a water-soluble calcium salt of sugar acids. One of these acids was proposed to be 2-keto-gulonic acid by the analyses of the calcium salt and melting point of the quinine salt.

L-Ascorbic acid was isolated and identified as a product resulting from the lactonization of the sugar acids that were obtained by the oxidation of L-sorbose by means of nitrogen tetroxide.

The oxidation of methyl-L-sorbopyranoside under anhydrous conditions gave, after lactonization, a strong dye-reducing (2,6-dichlorophenol-indophenol) substance and presumably is comparable to results obtained by the oxidation of L-sorbose under comparable conditions.

Sucrose was oxidized under conditions comparable to the oxidation of L-sorbose, and after acid treatment (lactonization), a strong dye-reducing (2,6-dichlorophenol-indophenol) substance was obtained.

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Amine Derivatives of Cyanuric Chloride*,†

By WILLIAM O. FOYE and ALBERT E. BUCKPITT‡

A number of new substituted amino, chloroamino, and hydroxyamino derivatives of cyanuric chloride have been prepared. Various methods of achieving the condensations were attempted.

SINCE the discovery that triethylene melamine and similar compounds containing the triazine ring have a growth-retarding effect on cancer cells in rats (1), it has appeared desirable to prepare and test other amino triazines. A variety of compounds of this type had already been tested for possible antimalarial effect (2) without success, however, but amino triazine derivatives of arsanilic acid have shown powerful therapeutic properties against spirochaetes and trypanosomes (3). The present investigation is intended to make available for testing other amino triazines with such pharmacologically active groups as the aminothiazole, diethylaminoethylamine, aminopyridine, and diaminodiaryl sulfone.

The methods which have been employed for preparing amino triazines have generally involved the treatment of cyanuric chloride with either a molar excess of the amine in an organic solvent or an equivalent of the amine in aqueous media with an inorganic base to neutralize the liberated hydrogen chloride. The second procedure ordinarily gives better yields, but suffers somewhat

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† This paper is abstracted from a thesis presented to the Graduate School of the University of Wisconsin by Albert E. Buckpitt in partial fulfillment of the requirements for the degree of Master of Science, September, 1951.
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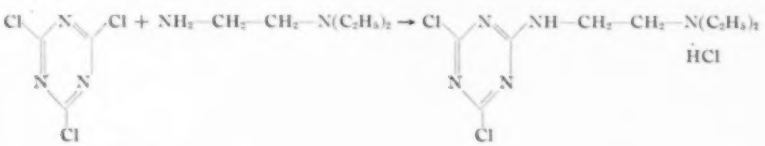
from the disadvantage that the products may undergo partial hydrolysis on drying or standing.

To avoid this, an attempt was made to prepare the amino triazines in organic media using an equivalent of the amine, with the addition slow enough so that the condensation product would accept the hydrogen chloride. On adding 2-aminothiazole to an acetone solution of cyanuric chloride, however, only mixtures of condensation product and 2-aminothiazole hydrochloride were obtained. This was indicated by the absence of a sharp melting point and a positive diazotization test for primary amine, since the analytical values of the thiazoleamino triazine hydrochloride and the mixture would be identical. When diethylaminoethylamine was reacted in acetone with cyanuric chloride, however, amino triazine hydrochlorides were obtained with sharp melting points



and negative nitrous acid tests for primary amine. The products were obtained in a good state of purity and gave correct nitrogen analyses without recrystallization. It is assumed that the presence of the tertiary amino group accounts for the acceptance of hydrogen chloride.

Another attempt to avoid aqueous media made use of pyridine as hydrogen chloride acceptor. When pyridine was added to an acetone solution of cyanuric chloride, a precipitate of the pyridinium salt was formed immediately, three equivalents of pyridine being taken up in this manner. The salt was hydrolyzed rapidly in water and was insoluble in organic solvents, and therefore was unsuitable for alkylation of amines.

It was necessary then to employ one of the previously used procedures, and the method of Thurston (4), using water as solvent with alkali as acid acceptor, was essentially followed. The amines were condensed with cyanuric chloride replacing either one, two, or all of the halogen atoms, depending upon the temperature of the reaction (5), and the products were formed in good purity and fairly good yields. Although some of the compounds could not be recrystallized, they gave a satisfactory nitrogen analysis by the micro-Kjeldahl method after being thoroughly washed with boiling acetone. The 2,4-dichloro-6-aminotriazines, however, gave evidence of partial hydrolysis after standing in screw-capped vials for several months. A majority of the compounds showed no melting or decomposition below 300° (see Table I).

EXPERIMENTAL

Substituted Amino Triazines.—A hot solution of cyanuric chloride (5 Gm.) in acetone or dioxane (40 ml.) was filtered and poured in a fine stream into water (60 ml.) cooled to 0° at such a rate that the temperature did not exceed 5°. To the slurry thus formed was added an equivalent amount of amine, and to the stirred reaction mixture an excess of aqueous sodium bicarbonate was added gradually. The reaction was stirred for a period of one-half to three hours, the temperature for a monoaminodichloro triazine kept at 0–5°, for a diamino-monochloro triazine kept at 40–45°, and for a triamino triazine, 100–125°. The precipitate was then filtered (boiling acetone may be necessary for precipitation), washed free of chloride ion with water, and pressed as dry as possible. After drying in a vacuum desiccator, the product was recrystallized from an appropriate solvent or washed well with several portions of boiling acetone. The compound was then dried in an oven at 60° for twenty-four hours.

Hydroxy Amino Triazines.—The monochloro diamino triazines or dichloro monoamino triazines were refluxed in an excess of either methanol or acetic acid for four hours. The solution was cooled and filtered, the solvent was distilled, and the residual solid was recrystallized from methanol. The compounds were then dried in an oven at 60° for twenty-four hours.

Diethylaminoethylaminotriazine Hydrochlorides.—Cyanuric chloride was dissolved in acetone, chloroform, or nitrobenzene, filtered, and cooled or heated to the appropriate temperature, depending upon the extent of condensation desired. The solution was treated slowly with the equivalent amount of freshly distilled diethylaminoethylamine dissolved in the same solvent, an hour being required for 2 Gm. of amine to be added. After stirring or refluxing for an additional hour, the solvent was distilled or the product filtered, and the solid dried *in vacuo*. The product was recrystallized from the appropriate solvent and dried *in vacuo* at an elevated temperature.

2,4 - Dichloro - 6 - (2,4′ - diamino - 5 - thiazolylphenyl sulfone) Triazine.—Cyanuric chloride (2.2 Gm.) was slurried in 60 ml. of water and 40 ml. of acetone in the described manner, and to the cooled (0°) mixture were added sodium bicarbonate (1 Gm.) and 2,4′-diamino-5-thiazolylphenyl sulfone (Promizole) (3 Gm.). The temperature was maintained at 0°, and the mixture was stirred for eight hours, at the end of which time the pale yellow precipitate was filtered and washed free of chloride ion.

TABLE I.—SUBSTITUTED AMINO TRIAZINES

R^1	R^2	R^3	M. P.	Crystallized From	Yield, %	Formula	Nitrogen Anal., % Calcd.	Found
2-Thiazolylamino	Cl	Cl	..	....	70	$C_6H_3Cl_2N_5S$	28.2	28.3
2-Thiazolylamino	OH	OH	..	Methanol	84	$C_6H_5O_2N_5S$	33.3	33.1
2-Thiazolylamino	2-Thiazolylamino	Cl	..	....	62	$C_9H_6ClN_7S_2$	31.5	31.2
2-Thiazolylamino	2-Thiazolylamino	OH	..	Methanol	82	$C_9H_7ON_7S_2$	33.4	33.1
2-Thiazolylamino	2-Thiazolylamino	2-Thiazolylamino	..	....	70	$C_{12}H_9N_9S_3$	33.7	33.9
Diethylaminoethylamino.-HCl	Cl	Cl	200–201°	Ethanol	60	$C_9H_{16}Cl_2N_6$	23.3	23.4
Diethylaminoethylamino.-HCl	OH	OH	263–265°	Ethanol	53	$C_9H_{18}O_2ClN_5$	26.6	26.2
Diethylaminoethylamino.-HCl	Diethylaminoethylamino.-HCl	Diethylaminoethylamino.-HCl	260–261°	Ethanol-Acetone	58	$C_{21}H_{46}Cl_3N_9$	23.7	23.8
2-Pyridylamino	Cl	Cl	258–260°	....	80	$C_8H_5Cl_2N_5$	29.1	29.4
2-Pyridylamino	2-Pyridylamino	Cl	238–240°	Water	50	$C_{13}H_{10}ClN_7$	32.8	32.6

The filter cake was pressed as dry as possible, and dried *in vacuo*. After recrystallization from acetone, 4 Gm. (73%) of product was obtained which showed no decomposition or melting below 300°.

Anal.—Calcd. for $C_{12}H_9O_2Cl_2N_9S_2$: N, 20.8. Found: N, 20.4.

SUMMARY

1. The preparation of amino derivatives of cyanuric chloride was found to require the presence of either alkali or a molar excess of amine to accept hydrogen chloride, except where a diamine is used as condensing agent.

2. The following new aminotriazines have been prepared: 2,4-dichloro-6-(2-thiazolylamino)-triazine, 2,4-dihydroxy-6-(2-thiazolylamino)-triazine; 2-chloro-4,6-di-(2-thiazolylamino)-triazine; 2-hydroxy-4,6-di-(2-thiazolylamino)-triazine; 2,4,6-tri-(2-thiazolylamino)-triazine; 2,4-dichloro-6-(diethylaminoethylamino)-triazine hydrochloride; 2,4-dihydroxy-6-(diethylaminoethylamino)-triazine hydrochloride; 2,4,6-tri-(diethylaminoethylamino)-triazine hydrochloride; 2,4-dichloro-6-(2-pyridylamino)-triazine; 2-chloro-4,6-di-(2-pyridylamino)-triazine; 2,4-dichloro-6-(2,4′-diamino-5-thiazolylphenyl sulfone)-triazine.

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The Amperometric Titration of Bismuth with 8-Hydroxyquinoline and Its Application to Compounds of Medicinal Importance*

By DALE W. BLACKBURN† and JOHN E. CHRISTIAN

A graphical amperometric end point using the dropping mercury electrode was applied to the direct titrimetric precipitation of dilute solutions of bismuth with a standard 8-hydroxyquinoline titrant in an acidic acetate buffer solution. The titration can be completed in forty-five to seventy-five minutes, and the accuracy is ±0.3 to ±0.8 per cent for bismuth concentrations between 0.001 and 0.01 *M*. A developed method was applied to various representative bismuth preparations and compared with the official gravimetric procedure.

THE AMPEROMETRIC METHOD, as defined by Kolthoff and Lingane (1), consists of the measurement of a change in the current, passing through the titration cell between an indicator electrode and a suitable depolarized reference electrode, as a function of the volume of the titrant. The titration is not affected by relatively large amounts of an indifferent electrolyte, and the graphical location of the end point increases the accuracy and eliminates a large number of readings near the end point. The amperometric method is as sensitive as the polarographic method of analysis, and increases its scope by being applicable to the determination of a polarographically inert substance as long as a suitable electro-active titrant is available. The use of the dropping mercury electrode instead of the rotating platinum electrode as an indicator electrode in the measurement of the bismuth diffusion current has the advantage of a higher hydrogen overvoltage.

Conn (2) used the dropping mercury electrode and the Electrochemograph in the microtitration of various medicinal diamidines, while Elofson and Mecherly (3) applied them to the amperometric titration of various couplers with several diazotized aromatic amines. The recording of the current allowed a more accurate measurement of the diffusion current and permitted one to study the reaction through the change in the current-voltage curves. Mader and Frediani (4) recently applied the rotating platinum electrode to the amperometric titration of halides in various pharmaceutical preparations. Gillis, Eeckhout, and Standaert (5) determined bismuth by direct amperometric titration with oxine in an acetate buffer. They used tartrate to form a soluble bismuth salt at a *p*H of 5.5 to 6.5.

8-Hydroxyquinoline or oxine is a selective organic precipitant which has been used frequently as an alternate gravimetric method for the analysis of various metals. In the titration of bismuth with oxine, three moles of the reagent react with one of bismuth by the replacement of the hydrogen of the hydroxyl group with bismuth to form the insoluble bismuth oxyquinoline (hydroxyquinolate).

In this work the diffusion current of bismuth was shown to decrease in value as a standard oxine solution was added and approached the residual current of the supporting electrolyte at the end point.

EXPERIMENTAL

Apparatus.—A Heyrovsky polarographic cell, having a mercury anode, was used in the preliminary polarographic analysis of bismuth in various supporting electrolytes. No specific temperature control was applied to either the polarographic analysis or the amperometric titration.

The titration cell is illustrated in Fig. 1. A length of barometer capillary tubing was used as the dropping mercury electrode, and a saturated calomel electrode was used as an external depolarized reference electrode. The calomel cell was attached to the titration vessel through a bridge of a 3% agar gel saturated with potassium nitrate. The end of the bridge entered a well filled with a saturated potassium nitrate solution. The liquid junction potential of the latter was eliminated with a sintered glass disk covered with a short length of a 3% agar gel saturated with potassium nitrate. A glass tube, filled with mercury and having a platinum seal, was inserted into the calomel flask to make an electrical contact between the lower mercury layer and one of the leads from the polarograph. Two nitrogen inlets, at the left of the dropping mercury electrode and passing through a No. 12 rubber stopper, were used to pass nitrogen into or over the solution. A Koch 10-ml. microburette was connected to a fine-bore burette tip by means of Z-shaped glass tubing with standard taper joints. A thin glass rod was attached to the burette tip to increase the accuracy of the burette readings. A Berzelius 200-ml. beaker was used as the titration

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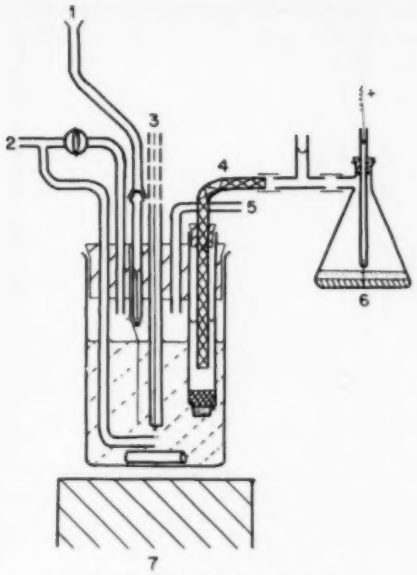

Fig 1.—Amperometric titration cell.
1. To a 10-ml. Koch microburette. 2. Nitrogen inlet. 3. To the dropping mercury reservoir. 4. Agar bridge. 5. Nitrogen exit. 6. Saturated calomel electrode. 7. Magnetic stirrer.

vessel, and the nitrogen passed out of the cell through a lip on the beaker or through an exit-tubing in the rubber stopper. A magnetic stirrer was placed 2 to 3 cm. below the vessel to thoroughly mix the reactants and to prevent the heating of the solution by the magnetic stirrer. A magnetic stirring rod was placed in the beaker before the addition of the bismuth solution. A water trap was placed between the titration cell and the nitrogen tank to saturate the inert gas with water vapor.

The Sargent Model XX Polarograph was connected to the dropping mercury electrode and the calomel electrode to study the change in the bismuth current-voltage curves and to record the diffusion current at a constant potential during the titration.

Development of the Method.—The reagents were standardized, and the current-voltage waves of bismuth in various supporting electrolytes were studied before the titration of known bismuth solutions with oxine. The oxine was a Paragon reagent grade product and was standardized by the back titration of an excess of standard potassium bromate with standard sodium thiosulfate in an acidic medium containing potassium bromide and iodide. The precision was $\pm 0.2\%$ and could not be improved upon by the use of lower temperatures ($-2°$ and $16°$). The known bismuth solutions were prepared by the addition of the supporting electrolyte to an aliquot of a bismuth stock solution (0.05 to 0.1 *N*), which was prepared by dissolving either Baker's bismuth lump metal or Mallinckrodt's analytical reagent grade of bismuth nitrate pentahydrate in enough concentrated nitric acid to give 0.5 to 1.0 *N* nitric acid upon dilution with distilled water. Bismuth subnitrate precipitated from 0.1 *N* nitric acid upon standing. The solid chemicals used in the supporting electolytes were either an analytical reagent grade or a reagent grade product. The mineral acids, ammonia, gelatin, and phenolphthalein were either the U. S. P. or N. F. grade. The polarographic mercury was washed with 10% nitric acid and was vacuum-distilled in an all-glass distillation unit (6).

The concentration of bismuth was held constant, and the quality of the bismuth current-voltage wave was compared in the common mineral acids (nitric, sulfuric, and hydrochloric) and acidic buffers (citrate, acetate, and tartrate) used as supporting electrolytes. As a result of this study, the acidic acetate buffer of Gillis, Eeckhout, and Standaert (5) was used as the supporting electrolyte in the titration of 4×10^{-3} *M* bismuth with 2% oxine in 1.0 *N* acetic acid. The diffusion current of bismuth decreased in value in proportion to the amount of oxine that was added. After the current in microamperes (corrected for dilution) had been plotted against the volume in milliliters of the titrant, the experimental end point agreed with the theoretical value within 0.3%.

In order to obtain the optimum conditions for the determination of bismuth by this method, the following factors were varied independently of each other: concentration of bismuth, oxine, acetate or tartrate; normality ratio of oxine to bismuth; buffer cation; stirring time and number of current readings; drop time and sensitivity; constant potential; maximum suppressor; and nitrogen rate. The maximum buffer *p*H of the acetate-supporting electrolyte was approximately 6.6. The following results were obtained:

1. The optimum concentration range of bismuth was between 1×10^{-3} and 1×10^{-2} *M*. The results were 0.8% low for 1×10^{-2} *M* bismuth, and 0.3 to 1.0% high for concentrations between 1×10^{-3} and 4×10^{-3} *M*.

2. The concentration of oxine was not critical but depended on the concentration of bismuth. The normality ratio of oxine to bismuth was varied between 10 and 20 to minimize the dilution effect.

3. A critical amount of tartrate was required to complex the bismuth and thus prevent its precipitation in the acetate buffer. Large amounts of tartrate prevented the precipitation of bismuth oxyquinoline and gave high results. The use of tartaric acid as a source of the tartrate ion decreased the amount of tartrate needed to solubilize the bismuth. Approximately 0.02 to 0.03 *M* tartaric acid was required to solubilize 3×10^{-3} *M* bismuth in a 0.40 *M* sodium acetate solution.

4. The concentration of acetate was not critical since a 30% reduction of its concentration did not affect the results.

5. The ammonium ion could be substituted for the sodium ion in the supporting electrolyte without affecting the results.

6. A smaller number of current readings (7 to 8) and a proportional increase in the stirring time (total of five to eight minutes) increased the precision of the assay. The current readings of the original sample, and frequently of the first addition of titrant, were low and were of no value in extrapolating the end point. The solubility of bismuth and oxine was generally unstable during the

first part of the titration, and the solution had to be stirred for a greater length of time. The titration points had to be 15–20% removed from the end point; otherwise the slight solubility of bismuth oxyquinoline gave a high current value.

7. The variance of the drop time between 3.3 and 2.3 seconds increased the sensitivity but did not affect the results. The sensitivity setting on the instrument should be adjusted to give a maximum current deflection for the original solution.

8. The constant potential of −0.9 volt could be reduced to −0.6 volt without affecting the results, but could not be increased since the characteristic hump or dip in the bismuth wave at approximately −1.0 volt gave irregular and scattered results. The half-wave potential of bismuth was approximately −0.3 volt, and the current-voltage wave consistently shifted to more positive potential values as the titrant was added because of the slight decrease in the *p*H of the buffer. The *p*H of the original sample was between 6.2 and 6.6, and decreased to between 5.1 and 5.5 after the addition of 9 to 16 ml. of the titrant.

9. Phenolphthalein was required to suppress the bismuth maximum.

10. The presence of oxygen because of an insufficient rate of nitrogen gave results that could not be reproduced and were approximately 4% high even though a good amperometric curve was obtained (Fig. 2). A nitrogen flow of 3.7 ml. per second was required for accurate results.

Application of the Method.—The necessary conditions for the accurate and precise determination of various known bismuth solutions having been obtained, the same conditions were applied to the determination of several official bismuth preparations. The buffer electrolyte of bismuth was prepared in the following manner. Approximately 3 Gm. of tartaric acid (0.04 *M*) was dissolved in an aliquot of a nitric acid solution of the bismuth unknown contained in a 250-ml. beaker, and the solution was neutralized with 25% sodium hydroxide using one drop of 0.1% methyl red as the indicator. The contents of the beaker were washed into a 500-ml. volumetric flask, and 16.41 Gm. of anhydrous sodium acetate (0.40 *M*), 10 drops of phenolphthalein T. S., and 4.0 ml. of 1.0 *N* acetic acid were added before being diluted with distilled water. One hundred milliliters of the bismuth sample (representing approximately 4×10^{-3} *M* bismuth) was pipetted into the titration vessel, and the solution was stirred with a magnetic stirrer and a flow of nitrogen (3.7 ml. per second) for fifteen minutes. One to two milliliters of the titrant, containing 0.1378 *M* oxine in 1.0 *N* acetic acid, was slowly run into the bismuth solution, and the solution was stirred for an additional four minutes. The magnetic stirrer was turned off, and the nitrogen was bypassed over the solution. After an interval of two minutes, the current was recorded for two divisions on the polarographic chart, and the recording pen was manually turned back to the original vertical line. The average diffusion current in millimeters of chart deflection for each addition of the titrant was corrected for dilution. The current was converted to microamperes by the use of a polarographic sensitivity chart, and plotted against the volume of titrant in milliliters. The experimental end point was graphically obtained from the intersection of two straight lines drawn through the plotted values.

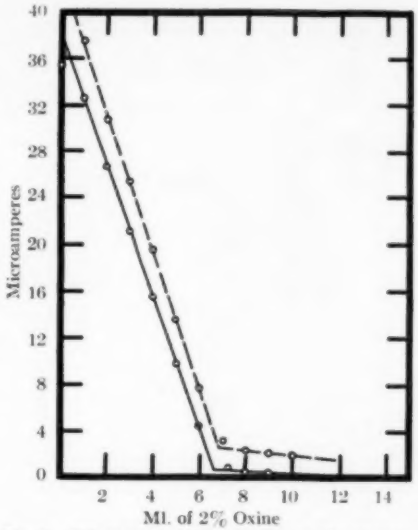

Fig. 2.—Effect of the nitrogen rate on the titration of 3×10^{-3} *M* bismuth.

– – – –, slow nitrogen flow; ———, rapid nitrogen flow. Theoretical end point = 6.53 ml.; slow nitrogen end point = 6.91 ml.; rapid nitrogen end point = 6.63 ml.

The results of the amperometric method for determining various official bismuth compounds are compared with the official gravimetric procedure in Table I. In the amperometric analysis of bismuth and potassium tartrate, the bismuth tartrate complex was split with 25% nitric acid. The U. S. P. XIV (7) does not state that the bismuth and potassium tartrate should be dried before the analysis. As the average loss on drying at 105° for three hours was 4.10%, the use of a dried sample is suggested to eliminate a possible source of a determinate error in the assay procedure.

In the amperometric analysis of bismuth subcarbonate, the salt was dissolved in 25% nitric acid with the aid of heat before its determination. The ignition assay of bismuth subcarbonate by the official U. S. P. method (7) was performed with a muffle furnace at approximately 950°.

In the amperometric analysis of the milk of bismuth, the magma was dissolved in 25% nitric acid before the analysis. The results were slightly above the official limit. The magma was evaporated to dryness in a 3-in. evaporating dish before ignition according to the N. F. IX method (8).

In the amperometric analysis of bismuth subgallate, the compound was digested with concentrated hydrochloric acid to decompose the basic salt. The bismuth was then separated from the gallic acid by precipitation of the sulfide. The bismuth sulfide was dissolved in hot dilute nitric acid after filtration, and the hydrogen sulfide was driven off by boiling the solution. In the N. F. method (8), the nitric acid solution of the ignition

TABLE I.—COMPARISON OF AMPEROMETRIC AND OFFICIAL METHODS FOR THE DETERMINATION OF SEVERAL OFFICIAL BISMUTH COMPOUNDS

		Official			Amperometric		
Compounds[a]	Official Limit[b]	No. of Assays	Mean	Standard Error of Mean	No. of Assays	Mean	Standard Error of Mean
Bismuth and potassium tartrate	60–64 Bi	4	60.84	$\pm 0.06_8$	4	61.46	±0.10
Bismuth subcarbonate	>90 Bi_2O_3	3	91.10	$\pm 0.01_8$	5	92.55	±0.20
Milk of bismuth	5.2–5.8 Bi_2O_3	3	5.67	$\pm 0.00_7$	4	5.86	$\pm 0.00_4$
Bismuth subgallate	52–57 Bi_2O_3	5	55.88	±0.61	6	54.00	±0.60

[a] Commercial samples.
[b] All figures are in per cent.

residue was evaporated to a syrupy consistency with an infrared lamp, and the remaining water and nitric acid were driven off with a low flame before reignition.

DISCUSSION

The reaction between bismuth and oxine to form bismuth oxyquinoline in a suitable acidic acetate buffer has been made the basis of a rapid and simple amperometric titration by the use of the dropping mercury electrode as the indicator electrode. The precision is ±0.4% and the accuracy is ±0.6% with bismuth concentrations between 1×10^{-3} and 1×10^{-2} *M*. The method has been applied to the analysis of bismuth and potassium tartrate, bismuth subcarbonate, milk of bismuth, and bismuth subgallate. The present official methods for the first three compounds were more accurate and precise, but the amperometric method was simpler and takes approximately two hours less time than the official methods. The accuracy and precision of the amperometric method is comparable to the official procedure for the assay of bismuth subgallate. The amperometric method is more sensitive than the usual gravimetric method of analysis and can be used to determine small concentrations of bismuth.

The bismuth sample must be air-free, and the concentration of tartrate is critical for obtaining accurate and precise results. The constant potential of −0.9 volt had been most frequently used, although a potential as low as −0.6 volt gave excellent results and also reduced the number of possible interferences.

It is suggested that a dried sample be used in the official assay of bismuth and potassium tartrate to eliminate a possible determinate error if the commercial products vary in their moisture content.

SUMMARY

1. An amperometric titration cell, containing the dropping mercury electrode and saturated calomel electrode and using a polarograph, was constructed.

2. The conditions affecting the amperometric titration of known bismuth solutions with standard oxine were determined.

3. A developed method of titration was applied to various representative bismuth preparations and compared with the official gravimetric procedures.

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An Analytical Application of Valser's Reagent

I. A General Method for the Assay of Organic Bases in Certain Pharmaceutical Products*

By RICHARD H. ROBINSON†

A nephelometric method for the detection and estimation of small amounts of methylhomatropine, thiamine, thenylpyramine, dihydrocodeinone, and strychnine in pharmaceutical products, is presented. The method uses common laboratory reagents and apparatus, is convenient, and may be applied to the assay of compounds which form insoluble iodomercurate complexes under the conditions of the method.

THE insoluble iodomercurate complex formed by adding Valser's reagent to slightly acidic, aqueous solutions of certain compounds has been of interest to us as a possible assay method for pharmaceutical preparations. Wachsmuth (1) has proposed an iodometric assay of thiamine based on the isolated iodomercurate complex. The large conversion factor involved enabled this worker to measure small amounts of the vitamin.

In an attempt to apply the Wachsmuth method to the assay of homatropine methylbromide (HMB), the persistence of the colloidal suspension of the iodomercurate complex was noted. A procedure based upon this finding gave accurate analytical results, even in the presence of aluminum salts and other organic compounds which give soluble iodomercurate complexes.

Bandelin (2) determined homatropine methylbromide by precipitating a reineckate complex, which was then dissolved in acetone and measured colorimetrically. We have found that aluminum sulfate interferes in the reineckate procedure, and tropanol methylbromide (a hydrolysis product of homatropine methylbromide) will yield the same amount of color when complexed with ammonium reineckate.

The absorbancies of aqueous suspensions of methyltropanol-iodomercurate and methylhomatropine-iodomercurate complexes differ sufficiently to allow measurement of the degree of hydrolysis of homatropine methylbromide. This observation will be expanded in a subsequent paper on the stability of homatropine methylbromide in pharmaceutical products.

The solubility of the iodomercurate complex of the compound to be assayed determines the usefulness of the method. For example, thenylpyramine or dihydrocodeinone are frequently combined in preparations with ephedrine, but, due to the solubility of the ephedrine iodomercurate complex, each can be assayed in the presence of ephedrine. Aluminum salts have no effect on the solubility of the complexes examined, although large amounts of sodium chloride have a solubilizing effect. Typical curves obtained by plotting the absorbancy of the iodomercurate complex in solutions containing 10 to 300 γ of the compound *versus* concentration are shown in Fig. 1.

EXPERIMENTAL

Reagents

Valser's Reagent.—Dissolve 70.0 Gm. of mercuric iodide (HgI_2) and 50.0 Gm. of potassium iodide (KI) in 500 ml. of 0.1 *N* sulfuric acid. One milliliter of a freshly prepared 1% starch suspension, added to this solution, will indicate the appearance of free iodine, which can be removed by the addition of a few drops of 0.1 *N* sodium thiosulfate.

Standard Solution of Organic Base.—Dissolve 20.0 mg. of the free organic base, or the equivalent weight of its soluble salt, in 0.1 *N* sulfuric acid and dilute to a final volume of 100 ml. with 0.1 *N* sulfuric acid. It was found that solutions of the compounds examined in this acid concentration were stable for several months.

General Methods of Sample Preparation

Tablets.—An amount of powdered tablets equivalent to about 20 mg. of the compound to be determined is placed in a rubber-stoppered bottle with several large glass beads, 100 ml. of 0.1 *N* sulfuric acid is added and mechanically shaken for two hours, then allowed to stand overnight and shaken for one-half hour in the morning. After filtration, a 1.00-ml. aliquot is used for assay. This procedure may be varied by grinding the powder in a mortar with small aliquots of acid and filtering each portion into a volumetric flask.

Suspensions.—Filter enough of the suspension to give at least 20 ml. of clear filtrate. Transfer aliquots of the filtrate equivalent to about 0.2 mg. of the compound to colorimeter tubes. Determine the specific gravity of both the filtrate and the suspension. The ratio of these two figures will enable the calculation of the amount of compound in the suspension from the amount found in an aliquot of the filtrate.

Solutions.—An aliquot equivalent to about 0.2 mg. of the compound is transferred to a colorimeter tube, or, if too concentrated for accurate measure-

* Received December 8, 1951, from the Control Department, Maltbie Laboratories, Inc., Newark, N. J.
† The author is indebted to R. H. Barry for his advice and encouragement in this investigation.

TABLE I

Preparation	Labeled Amount	Amount Found
Suspension of aluminum hydroxide with homatropine methylbromide (HMB)	5 mg. HMB/fluid ounce	5.09 mg. 5.05 mg. 4.98 mg.
Elixir of phenobarbital with homatropine methylbromide	16 mg. HMB/fluid ounce	16.00 mg. 16.08 mg. 15.85 mg.
Ampuls of homatropine methylbromide	0.500% HMB	0.497% 0.501% 0.496%
Thiamine hydrochloride (B_1) tablets	5 mg. B_1/tablet	5.10 mg. 5.00 mg. 5.15 mg.
Elixir ferrous sulfate with thiamine hydrochloride	5 mg. B_1/fluid ounce with a 10% excess	5.65 mg. 5.58 mg. 5.70 mg.
Thenylpyramine hydrochloride with ephedrine hydrochloride tablets	0.375 grain thenylpyramine hydrochloride/tablet	0.380 Gr.
Syrup dihydrocodeinone bitartrate with ephedrine hydrochloride	0.1667 grain of dihydrocodeinone bitartrate/fluid ounce	0.173 Gr.
Strychnine sulfate tablets	0.0333 grain strychnine sulfate/tablet	0.0336 Gr.

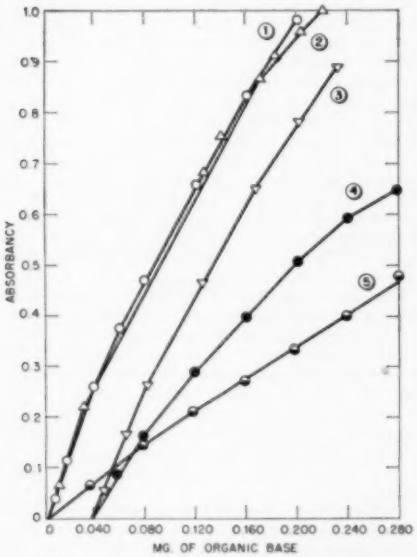

Fig. 1.—Concentration *versus* absorbancy.
1. Methylhomatropine. 2. Thenylpyramine. 3. Thiamine. 4. Dihydrocodeinone. 5. Strychnine.

ment, an aliquot is diluted with water in such a manner as to result in a solution containing 0.2 mg./ml.

Method

Aliquots containing 0.2 mg. of the compound to be determined are placed in colorimeter tubes and diluted with water to a total volume of 16 ml. Valser's reagent (1.00 ml.) is added and the tube is allowed to stand for at least five minutes but not more than fifteen minutes. Tubes containing exactly 0.200 mg. of the reference compound are precipitated with the same amount of Valser's reagent and allowed to stand for exactly the same amount of time. After standing, the absorbancies of the suspensions are read in a suitable instrument at an arbitrarily chosen wave length. The author used an Evelyn colorimeter and filter No. 515. A blank tube containing 16 ml. of water and 1 ml. of Valser's reagent is set at zero absorbancy. The effect of color present in the original preparation may be corrected as follows: after reading the absorbancies of the suspensions, a crystal of solid KI is added to the iodomercurate suspension, and dissolved. This excess KI dissolves the iodomercurate complex and enables the reading of residual color which is subtracted from the total absorbancy. Typical assay results on various pharmaceutical products are listed in Table I.

DISCUSSION

A calculation of the standard deviation, from data obtained with 25 determinations of known concentrations at the same time, shows an accuracy of ±3%. As the assay is of such simplicity, triplicate or even quadruplicate determinations have the advantage of increasing accuracy without greatly lengthening the assay.

SUMMARY

A method is presented for estimating small amounts of methylhomatropine, thiamine, thenylpyramine, dihydrocodeinone, and strychnine in pharmaceutical products by precipitating these bases as iodomercurate complexes and determining the absorbancies of the suspensions.

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The Quantitative Evaluation of Aromatic Waters*,†

By B. F. COOPER and E. A. BRECHT

A satisfactory method for the quantitative evaluation of aromatic waters was devised. The method was based upon the production of cloudiness in an aromatic water when a quantity of salt solution was added. By determining the quantities of a standard salt solution required to produce cloudiness in aqueous solution of a given oil, using various percentage saturations, reference curves are constructed by plotting percentage saturation against cubic centimeters of standard salt solution. These curves can be used for estimating the percentage saturation of aromatic waters of unknown saturation directly from measurements of the quantities of standard salt solution required to produce cloudiness. Sodium citrate was found to be the best salting-out agent.

HISTORICALLY, aromatic waters occupy a prominent role in pharmacy and medicine (1–4). So highly were aromatic waters valued that until the end of the 18th century the official pharmacopeias of all countries vied with each other in the number and variety of their formulas for distilled waters. Today they no longer occupy such a dominant position in therapeutics, but they have at least minor importance as therapeutic agents, vehicles, and flavoring agents (5–7). Since there are 12 official aromatic waters but no official means of analysis, there exists a definite need for a convenient method of evaluation.

Several methods for the quantitative evaluation of aromatic waters are described in the literature (8–19). The methods most commonly employed are: (*a*) extraction of the oil with a suitable solvent, (*b*) precipitation of the oil and measurement of its volume after separation by centrifugal force, and (*c*) measurement of the surface tension of the water.

These methods are not used because they are not accurate for the assay of the dilute concentrations of aromatic substances found in waters. They do not test the degree of saturation. They require a high degree of technique and the use of special equipment not usually available in pharmacies and small laboratories.

It is well known that the presence of salts in water generally decreases the solubility of aromatic substances. It was found that the addition of a saturated sodium chloride solution to an aromatic water produced cloudiness in the mixture by the precipitation of a portion of the aromatic substance. It was also found that more saturated salt solution was required to produce cloudiness if the aromatic water was diluted with distilled water. This evidence was developed into a practical method for assaying the quality of aromatic waters.

EXPERIMENTAL

Twenty salts were screened as precipitating agents. Saturated solutions of each were prepared and used to titrate 15-cc. samples of three aromatic waters: camphor water, peppermint water, and cinnamon water, representing a ketone, an alcohol, and an aldehyde, respectively. Three strengths (100%, 80%, and 60% saturated) of each water was titrated with each saturated salt solution. The two lower strengths were prepared by dilution of the saturated waters. The end point of cloudiness was best observed in a test tube held before a dark background and illuminated at a right angle to the line of vision by an electric lamp. All of the work in this paper was conducted at room temperature which did not exceed the limits of 25–28°.

From the titration data the 20 salts were rated in the order of decreasing efficiency as precipitating agents for aromatic waters: potassium citrate, calcium chloride, sodium citrate, magnesium sulfate, potassium phosphate, potassium sodium tartrate, aluminum chloride, sodium chloride, sodium sulfate, sodium acetate, sodium bromide, calcium bromide, ammonium chloride, potassium bromide, potassium chloride, ammonium bromide, potassium nitrate, sodium phosphate, potassium bitartrate, and sodium bitartrate.

There was no success in the attempt to correlate the gradation of efficiency on the basis of the following properties: pH, solubility, specific gravity of the solution, concentration, molecular weight, molar concentration, ionic concentration, and ion content.

Of the six best salting-out agents, two salts were eliminated from further study: potassium citrate, because the saturated solution was slightly colored; and potassium phosphate, because it is not official and, therefore, not generally available. The other four salts, calcium chloride, sodium citrate, magnesium sulfate, and potassium sodium tartrate were tested further by the titration of the remaining official aromatic waters. On this basis, sodium citrate was rated as the best salting-out agent.

None of the salts produced cloudiness when added to rose water, stronger rose water, orange flower water, and hamamelis water. This was anticipated in the cases of rose water (because it is at

* Received August 31, 1951, from the School of Pharmacy, University of North Carolina, Chapel Hill.
Presented to the Scientific Section, A. PH. A., Buffalo meeting, August, 1951.
† Abstracted from a thesis submitted to the Graduate School of the University of North Carolina by B. F. Cooper as partial fulfillment for the degree of Master of Science.

best only 50% saturated) and hamamelis water (because it contains alcohol). In the case of orange flower water and stronger rose water the samples were of poor quality. Due to a lack of materials, these two waters were not freshly prepared, but it is considered probable that this method of analysis is applicable to them.

Saturated sodium citrate solution was then used to titrate in triplicate each of eight official aromatic waters and dilutions of them. The averages are given in Table I, and the curves from these data are shown in Figs. 1–7. Chloroform water is omitted because the high volatility of chloroform makes it difficult to obtain a reproducible end point and because the official chloroform water contains excess solute to insure complete saturation.

Experience with this method increases accuracy in finding a reproducible end point of cloudiness. For very accurate determinations, it is recommended that each analyst prepare his own titration curves. For less accurate work, the curves appearing in Figs. 1–7 are sufficient.

The accuracy of the method for the assay of a particular sample depends upon the solute and its degree of saturation. A statistical analysis of the complete data showed that the accuracy decreases in

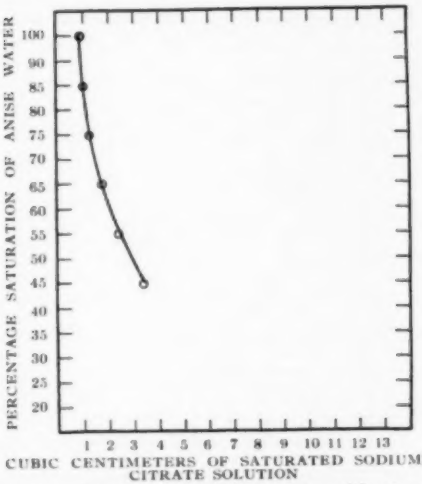

Fig. 1.—Titration curve of anise water with saturated sodium citrate solution.

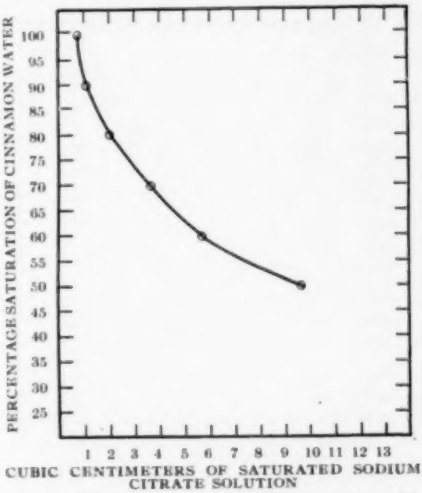

Fig. 3.—Titration curve of cinnamon water with saturated sodium citrate solution.

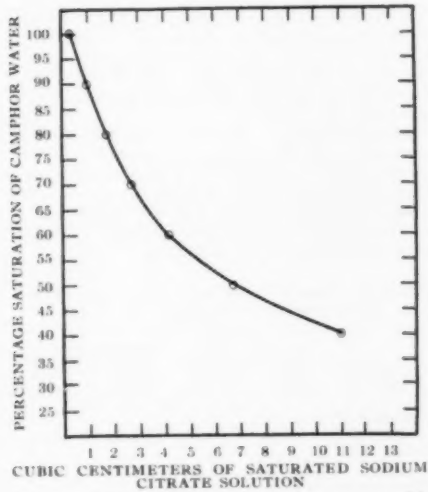

Fig. 2.—Titration curve of camphor water with saturated sodium citrate solution.

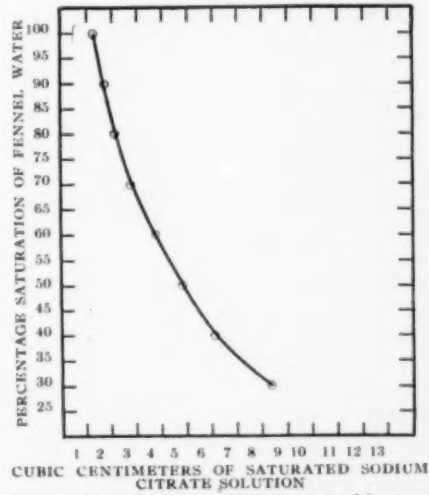

Fig. 4.—Titration curve of fennel water with saturated sodium citrate solution.

TABLE I.—VOLUME OF SATURATED SODIUM CITRATE SOLUTION REQUIRED TO PRODUCE CLOUDINESS IN 15-CC. SAMPLES OF AQUEOUS SOLUTIONS OF VOLATILE CONSTITUENTS

Saturation, %	Saturated Sodium Citrate Solution Required, Cc.						
	Anise Water	Camphor Water	Cinnamon Water	Fennel Water	Peppermint Water	Spearmint Water	Wintergreen Water
100	0.90	0.32	0.72	1.34	1.09	0.67	0.88
90	1.04	0.97	1.16	1.76	1.66	1.11	1.35
80	1.28	1.79	2.04	2.17	2.23	1.95	1.82
70	1.79	2.73	3.65	2.80	3.16	2.91	2.44
60	2.41	4.22	5.71	3.76	4.21	4.47	3.08
50	3.40	6.79	9.66	4.85	6.38	6.77	4.07
40	..	11.03	..	6.17	8.77	9.97	7.57
30	...	...	..	8.43	..	..	..

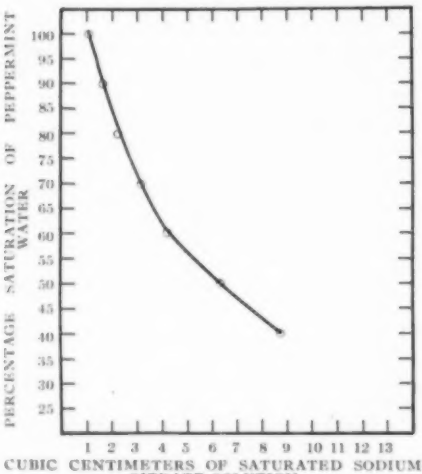

Fig. 5.—Titration curve of peppermint water with saturated sodium citrate solution.

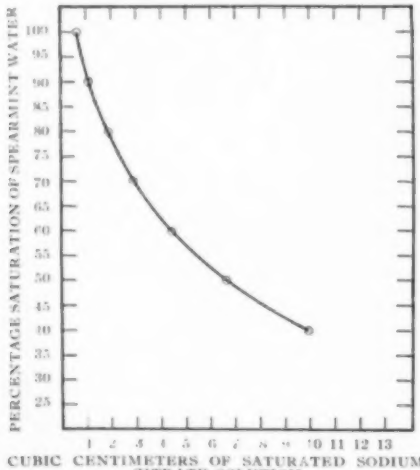

Fig. 6.—Titration curve of spearmint water with saturated sodium citrate solution.

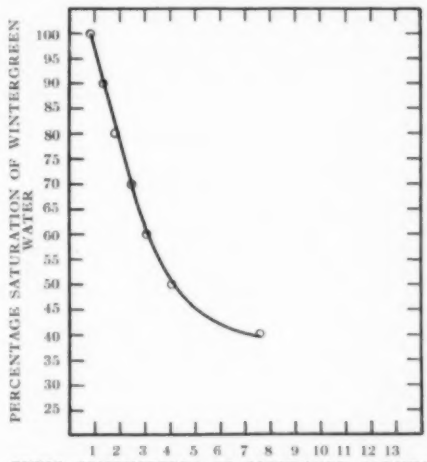

Fig. 7.—Titration curve of wintergreen water with saturated sodium citrate solution.

the following order: camphor, cinnamon, fennel, peppermint, spearmint, anise, and wintergreen. It also shows that the accuracy decreases as the degree of saturation decreases. The over-all variance was equal to 0.013 with a corresponding standard deviation of ±0.114.

Effect of Storage.—To observe the effect of various types of storage on preserving the quality of aromatic waters, three were stored for two months at room temperature in 16-oz. bottles. The data in Table II show that a cork closure is superior to a cotton closure, but there is no significant difference between colorless and amber bottles.

TABLE II.—PERCENTAGE SATURATION OF AROMATIC WATERS AFTER TWO MONTHS' STORAGE

Aromatic Water	Amber Bottle, Cork Closure, %	Amber Bottle, Cotton Closure, %	Colorless Bottle, Cork Closure, %	Colorless Bottle, Cotton Closure, %
Camphor water	100.0	75.5	100.0	65.5
Peppermint water	100.0	55.5	100.0	65.5
Wintergreen water	100.0	49.0	100.0	40.0

The effect of removing a quantity of aromatic water from the storage container at specified intervals is indicated in Table III.

TABLE III.—PERCENTAGE SATURATION OF AROMATIC WATERS DURING REMOVAL OF ONE OUNCE EVERY THREE DAYS

Aromatic Water	1st Day, %	12th Day, %	24th Day, %	36th Day, %	48th Day, %
Camphor water	100.0	100.0	99.0	98.0	98.0
Peppermint water	100.0	93.0	92.5	90.0	89.5
Wintergreen water	100.0	90.5	86.5	81.5	78.5

The evidence indicates that aromatic waters decrease in quality as the quantity in the storage container is decreased, the amount of decrease being dependent upon the particular aromatic water employed.

Qualitative Standards.—Standards of quality for aromatic waters can be established by utilizing the data contained in this paper. It is suggested that the minimum quality be set at 75% saturation. Table IV shows the quantities of saturated sodium citrate solution necessary to produce cloudiness in 15-cc. samples of 75% saturated aromatic waters.

TABLE IV.—VOLUME OF SATURATED SODIUM CITRATE SOLUTION TO PRODUCE CLOUDINESS IN 15-CC. SAMPLES OF 75% SATURATED AROMATIC WATERS

Aromatic Water	Saturated Sodium Citrate Solution, Cc.
Camphor water, U. S. P.	2.19
Cinnamon water, U. S. P.	2.82
Peppermint water, U. S. P.	3.15
Anise water, N. F.	2.00
Fennel water, N. F.	2.50
Spearmint water, N. F.	2.39
Wintergreen water, N. F.	2.20

If no cloudiness is produced upon the addition of the appropriate quantity of saturated sodium citrate solution, the aromatic water may be considered below standard.

SUMMARY

1. A satisfactory method for the quantitative evaluation of aromatic waters was devised.

2. There was a wide variation among salt solutions regarding salting-out efficiency, saturated sodium citrate solution being the most efficient. The attempt to correlate salting-out efficiency with chemical and physical properties was unsuccessful.

3. The accuracy of the proposed method depends upon the particular aromatic water employed and its percentage saturation, as indicated by the computation of the appropriate variances and standard deviations.

4. Aromatic waters stored over a two-month period kept equally well in amber-colored and colorless glass containers, but kept better with a cork closure than with a cotton closure.

5. Aromatic waters decreased in quality as the quantity in the storage container was decreased, the amount of decrease being dependent upon the aromatic water employed.

6. Standards of quality for aromatic waters were suggested.

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Note

A Note on a New Single Bottle Perfusion Unit*

By A. K. REYNOLDS†

THE UNIT shown in Figure 1 was designed to facilitate the procedure of carrying out continuous perfusions with varying concentrations of a drug. In the usual technique, perfusion with physiological saline solution on the one hand, and with drug solution on the other, is carried out from separate bottles containing the respective solutions. With such an arrangement, it is necessary to prepare another solution of the drug each time that perfusion with a higher concentration is desired. In addition, the perfusion must be interrupted, the bottle containing the drug solution opened, and the solution changed. The single unit system described here eliminates these procedures. The physiological saline solution is placed in a graduated Marriotte bottle of suitable capacity for the particular experiment. A concentrated solution of the drug of definitely known strength is placed in a reservoir (*R*) which can be made easily by cutting off the lower part of a burette. The entire unit consisting of drug reservoir (*R*), pressure regulating tube (*T*), and stirrer (*S*) is conveniently incorporated in the rubber stopper inserted in the Marriotte bottle. The drug reservoir, of course, must be closed to the external air, but since the *top* of the reservoir is connected with the Marriotte bottle by a length of tubing, drug solution will flow into the Marriotte bottle when the stopcock on the reservoir is opened.

Perfusion is started in the usual manner with the physiological saline solution. Perfusion with drug solution is then carried out by merely delivering from the reservoir the calculated amount required to make the desired final concentration. Rapid and thorough distribution of the drug is obtained by rotating the stirrer which is made from small-size glass rod or tubing, bent as shown to increase stirring efficiency. With this apparatus, the concentration of the drug in the perfusion

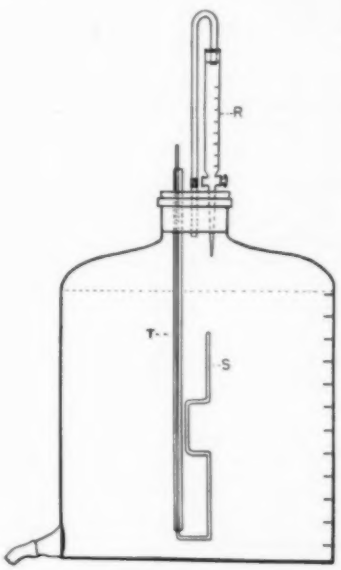


Figure 1

fluid may be quickly increased whenever desired, without interruption of the perfusion. By employing a second Marriotte bottle containing physiological saline solution only, and connected to the cannula with the usual Y-piece, a change to perfusion with this fluid alone may be obtained, if necessary.

* Received Febuary 11, 1952, from the Department of Pharmacology, School of Medicine, Dalhousie University, Halifax, Nova Scotia, Canada.

†Assistant Professor of Pharmacology, Dalhousie University.

Book Reviews

Arithmetic of Pharmacy. By CHARLES H. STOCKING and ELMON L. CATALINE. D. Van Nostrand Co., Inc., New York, 1952. xii + 148 pp. 23.5 x 16 cm. Price $2.75.

This book is based on the original text by A. B. Stevens, the seventh edition of which was revised by Stocking and Cataline. In the preparation of their *Arithmetic of Pharmacy*, the authors have rewritten and rearranged a considerable portion of the material, but at the same time have preserved the general pattern established in earlier editions of Stevens' book. Descriptive matter and illustrations have been used in the first five chapters to a greater extent than heretofore, and this should serve to clarify many of the procedures discussed. An entirely new chapter treating calculations especially related to the compounding of prescriptions has been added. The sections on certain basic arithmetical operations have been collected and appear in an appendix. This material should prove useful to students who have forgotten the fundamental operations of elementary arithmetic. This book has enjoyed many years of popularity as a teaching tool and as a textbook for beginning students in pharmacy. There is good reason to believe that the new edition of the *Arithmetic of Pharmacy* will maintain the position attained by its predecessors.

The binding and printing of the book are of good quality and the format has been modernized and made more attractive and readable than the previous editions.

The Mitotic Cycle. By ARTHUR HUGHES. Academic Press, Inc., New York, 1952. viii + 232 pp. Illustrated. 16 x 25.5 cm. Price $6.

In the foreword to this book, the author emphasizes that it is not a textbook of cytology, but that it has been written for readers possessing a knowledge of the elements of that subject. The intention of the book has been to review recent progress in the study of the dividing cell in which there has been a decided increase in interest during recent years.

In the first chapter the nucleic acids are discussed, because the author believes ". . . there should be given some account of those macromolecules the changes in which at a lower order of magnitude are now known to relate to the visible events within the cell, and are believed in some way to initiate and govern the course of the whole complex of biological events which results in the production of two cells from one."

The scope of the book is indicated by the titles of the other five chapters which are: The Interphase Cell and the Cytoplasm; The Interphase Nucleus; Cells in Division; Experimental Analysis; and Outlook.

The book is well documented with references to the original literature at the end of each chapter and it is provided with an author index and a subject index.

Scientific and Technical Abbreviations, Signs and Symbols. 2nd ed. By O. T. ZIMMERMAN and IRVIN LAVINE. Industrial Research Service, Dover, N. H., 1949. xiv + 541 pp. 21.5 x 15 cm. Price in U.S., $8; Foreign, $9.

This book was designed with two objectives in mind. One of these objectives was to make available in one convenient volume the principal abbreviations, signs, and symbols used in a number of scientific and technical fields. Another aim was to encourage greater uniformity in the use of abbreviations, signs, and symbols. These objectives are difficult to achieve, but certainly an excellent start toward them has been made. Most of the sections should be of interest to research workers in the pharmaceutical field because the interests of this group have so many ramifications. The abbreviations used in mathematics, statistics, chemistry, physics, medicine, botany, and zoology appear to be standard ones, and from time to time many abbreviations from these various classifications are used in scientific pharmaceutical journals. Quite a number of the abbreviations presented in tabular form are taken from recognized sources. The abbreviations used in chemistry, for example, are taken from *Chemical Abstracts*. It is truly a very great convenience to have nearly all scientific abbreviations and symbols in one book, and this one can be recommended as a useful reference to anyone preparing or editing scientific papers in the pharmaceutical field.

The National Formulary 1952. Published by the British Medical Association and the Pharmaceutical Society of Great Britain, London, 1952. 196 pp. 11 x 17 cm. Price 4*s.* 6*d.* (postage 3*d*).

This pocket-size handbook was first published in 1949 by a Joint Formulary Committee of the British Medical Association and the Pharmaceutical Society of Great Britain as a prescribers' formulary. The second edition includes enough additional material to increase the number of pages by more than fifty per cent.

The first section contains "Notes for Prescribers" and gives a concise review of the first-aid treatment of poisoning, and discussions of therapeutic classes of drugs. A pharmacological classification of drugs is also given.

The General Section of formulas groups the preparations according to pharmaceutical types, e.g., applications, capsules, etc. The names at the beginning of the subdivisions are given in English with the Latin in smaller type, but the names of the individual preparations are given first in Latin with the English name in a secondary role. Practically all the formulas are for products in the B. P. or B. P. C.

A special section is devoted to preparations for infants. All the mercurial preparations that were included in this section in the 1949 edition of the British N. F. were deleted in the 1952 edition.

Attention has been given to making the medicines for infants more attractive and palatable.

Proprietary preparations are listed along with preparations which (A) can be regarded as identical or (B) are substantially the same or are reputed to have analogous therapeutic effects. The A and B preparations are mainly B. P. or B. P. C. products.

In order to assist prescribers to recognize proprietary substances by names approved by the General Medical Council, a list of approved or official names, together with "other names" used for the same substances, is included. Since the idea is to acquaint the prescribers with the approved or official names for the proprietary products which are known by the other names, it would have better served the desired purpose to have a list of proprietary names with the approved name opposite. As the list is given, only in those cases where the spelling of the proprietary name is similar to the approved name can the relation be established readily when only the proprietary name is known. The "other names" are not included in the general index.

E. Merck's Jahresbericht. 64th yearly edition, 1950. Verlag Chemie G. m. b. H., Weinheim A. D. Bergstr., 1951. 432 pp. 14.5 x 22.3 cm.

This book, printed in German, gives concise reviews of therapeutic and pharmaceutic developments of old and new medicinal agents. The literature of 1950 is the source of the text material for this edition.

The substances reviewed are arranged in alphabetical order, but Latin names are used for some products, while German names are used for others. The first group of compounds discussed is acids: Acidum *p*-aminobenzoicum through Acidum undecylenicum. Several pages later, the Aminosäuren are presented. Trade-marked products are placed according to their trade names, the last item being: "Xylocain, das in Amerika die Bezeichnung 'Lidocain' führt." A general index and an index listing the drugs under the conditions for which they are therapeutically indicated are given.

This is a very good book to include in a reference library. The value of these volumes would be enhanced if they could be published earlier after the close of the period that is reviewed.

American Pharmacy. 3rd ed. RUFUS A. LYMAN, Editor-in-Chief. J. B. Lippincott Co., Philadelphia, 1951. xiii + 505 pp. Illustrated. 19 x 26 cm. Price $8.

This book is an excellent text for the student pharmacist, and it is a splendid source of refresher material for the practicing pharmacist. The many co-authors, practically all of whom are members of the same pharmaceutical generation, exhibit a similarity of style that permits the reader to pass from one subject to another without realizing that they were compiled by different men. Perhaps this is due to the fine editorship of Rufus A. Lyman. This edition of *American Pharmacy* continues the high standard of the two earlier editions of the book.

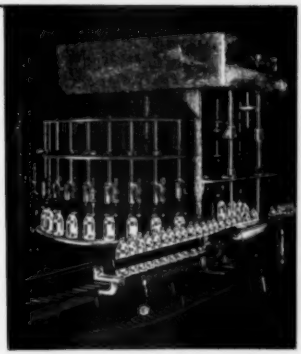
Correlation of official preparations and their trade-named equivalents is a very good feature of the text. However, the trade-name product similar to phenylephrine hydrochloride injection is Neo-Synephrine Hydrochloride Injection. Only Neo-Synephrine appears in the index, which also includes Solution Zephriran Chloride.

The revision of the text by selected active educators is reflected in the interesting presentation of the different divisions. In consideration of the amount of scientific pharmacy that is carried on in the laboratories and compounding sections of the drug manufacturers, it might be interesting to invite more representatives of the industrial pharmaceutical specialties to participate in the next revision of this fine book.

WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

4-Mercapto-2-phenylbenzothiazole
N-Methyl synephrine
Methadren
D-Threitol
Aneurine pyrophosphoric acid
Benzohydroxamic acid
Cadaverine
Calcium thiolactate
Codeohydrase-1
Cysteine sulfone
Cytochrome A
Dehydroascorbic acid
N,N-Diethyl propane-1,3-diamine
Dinucleotide adenoflavine
Glyoxalic acid
Guanazole
Indoxyl
Linoleyl alcohol
Lysergic acid
Phytase



Finer service for you through concentration

on fewer fine chemicals

One of our specialties:

ammonium chloride

A DUAL-PURPOSE CHEMICAL

You are served better and *in more ways* when we keep our production of fine chemicals to less than one hundred.

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Concentration enables us to offer Ammonium Chloride to you as a dual-purpose product. It is physically uniform—made in the proper range of particle size to permit direct compression into tablet form or to go into solutions quickly. This dual-purpose feature eliminates duplicate inventories for you, and helps reduce your production costs.

Concentration enables us to offer you an Ammonium Chloride whose chemical purity is precisely controlled from raw materials to finished product. It is produced in stainless steel equipment as an added safeguard to purity.

Whatever your requirements for Ammonium Chloride—call on Baker. Samples and prices will be forwarded gladly upon request. Address: *Fine Chemical Division*, J. T. Baker Chemical Co., Phillipsburg, N.J.

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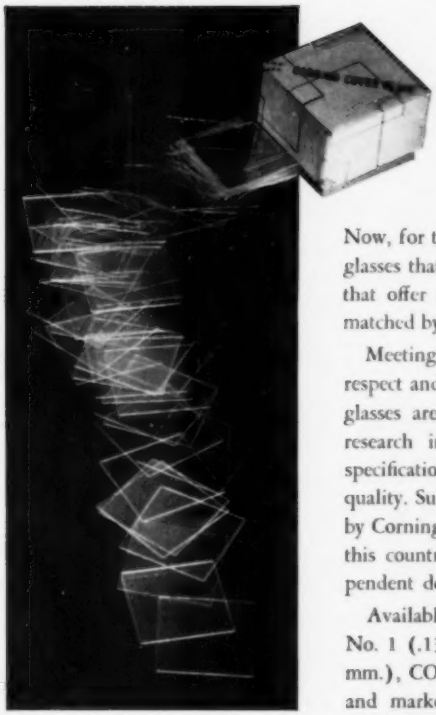
Announcing...

CORNING MICRO COVER GLASSES



Now, for the first time, you can get micro cover glasses that are water-white crystal in color and that offer a degree of flatness that cannot be matched by any other cover glass on the market.

Meeting government specifications in every respect and of finest optical quality, these cover glasses are a product of Corning's continuing research in glass. Manufactured to exacting specifications, they are uniform in thickness and quality. Successful production of this cover glass by Corning means that laboratory technicians in this country will now have a completely independent domestic supply of micro covers.

Available in two standard thickness ranges, No. 1 (.13 to .17 mm.) and No. 2 (.17 to .25 mm.), CORNING Micro Cover Glasses are cut and marketed by Alfred Bicknell Associates, Cambridge, Mass. and J. Melvin Freed, Inc., Perkasie, Pa. Your laboratory supply dealer carries complete stocks. Contact him today.

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